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GC-MS Analysis of the Secondary Metabolites from the Leaves of Wild Palestinian *Calamintha incana* and their *in-vitro* Antioxidant and Antimicrobial Activities

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M.Sc. Thesis

Jerusalem-Palestine

1439/2017

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A thesis submitted in partial fulfillment of requirements for the degree of Master of Applied Industrial Technology - Deanship of Graduate Studies -Al-Quds University.

1439/2017

Al-Quds University

Deanship of Graduate Studies

Applied Industrial Technology

Thesis Approval

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Master thesis Submitted and Accepted, Date: 14/10/2017

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Dedication

This thesis is dedicated to:

- My brother, ***the martyr Ahmad Jahajha***, who is immortal in our hearts.
- My family. A special feeling of gratitude to my loving parents, whose words of encouragement and push for tenacity to finish this thesis and to my brother and sisters who have never left my side and are very special.
- My dearest wife: for her endless support and encouragement.
- My beloved kids: Hassan, Mohammad, Abed Al-Majid and Ahmad, who lighten my life up and give me the power to keep on.
- My friends and work family who have supported me throughout the process and finishing this thesis.

Ali Hassan Ali Jahajha

Declaration

I certify that this thesis submitted for the degree of master is the result of my own research, except where otherwise acknowledged, and this thesis has not been submitted for the higher degree to any other university or institute.

Signed: 

Ali Hassan Ali Jahajha

Date: 14/10/2017

Acknowledgments

I would like to express my deep gratitude to Allah, the most compassionate and the most merciful, who enabled me to accomplish this Research.

I would like to thank all the people who contributed in some way to facilitate the success of the work described in this thesis. First and foremost, I am very grateful to my supervisor, Professor Dr. Saleh Abu-Lafi, for his supervision, useful comments and continuous support. I appreciate giving me the intellectual freedom to engage new ideas while demanding high quality of work in my research.

I would like to knowledge the Central Palestinian Health Laboratories to which this research was conducted. My deepest respect and thanks to my friends and colleagues for their valuable help and supporting me there.

Many thanks to my parents; your prayer was what sustained me thus far.

Finally, I am grateful to my loves one, my wife and children who inspired and encouraged me to explore the best in me. I thank them for dedication and patience.

List of Abbreviations

Abbreviation	**Full word**
AI %	Antioxidant scavenging activity
ATCC	American Type Culture Collection
BHT	tert-butyl-4-hydroxy toluene
CFU	Colony forming unit
Cm	Centimeter
C. incana	*Calamintha incana*
DMAPP	Dimethylallyl diphosphate
DPPH	2, 2'-diphenyl-1-picrylhydrazyl
DRI	Daily Required Intake
DXP	1-deoxy-d-xylolose-5-phosphate
EI	Electron Impact
EO's	Essential oils
EPA	Environmental Protection Agency
GC	Gas Chromatography
GCMS	Gas Chromatography-Mass Spectrometry
IAEA	International Atomic Energy Agency
IC_{50}	Inhibitory Concentration 50
ICP-OES	Inductively Coupled Plasma-Optical Emission Spectrometry
IPP	Isopentenyl diphosphate
KI	Kovats Index
IR	Infra-Red
LOD	Limit of Detection
LOQ	Limit of Quantitation
MEP	2-C-methylerythritol-4-phosphate
MS	Mass Spectrometry
MVA	Mevalonic acid
NIST	National Institute of Standards and Technology
RSD	Relative Standard Deviation
RT	Retention Time
SD	Steam Distillation
TIC	Total ion chromatogram
USA	United states of America
USP	United states Pharmacopeia
WHO	World Health Organization
β	Beta
µ	Micro

Abstract

The overall aim of the current study is to screen and determine the wild Palestinian *Calamintha incana (C. incana)* volatiles and semi volatiles secondary metabolites present in its leaves by using steam distillation (SD) and gas chromatography-mass spectroscopy (GC-MS). We hypothesize that such compounds may have potential to cure certain diseases. Therefore, the anti-oxidant, anti-microbial and anti-fungal activities were determined. Moreover, the metals content were also analyzed by using inductively coupled plasma-optical emission spectroscopy (ICP-OES).

The wild *C. incana* leaves essentials oils were first separated by SD followed by direct analysis using GC-MS in the electron impact mode (EI). Seventeen components were separated with high resolution and identified using the NIST library of the mass spectrometer and by comparing their Kovats index (KI) with authentic standards. The predominant components were pulegone, p-menthan-3-one and caryophylene oxide.

The antibacterial activity of the essentials oils was examined for various microorganisms using inhibition zone method by using the disc agar diffusion technique. Gentamicin, Ciprofloxacin and Nystatin were used as positive controls. Antioxidant activity was also measured by spectrophotometric method. 5 µl of the essentials oils demonstrated to have activity against *Staphylococcus aureus, Salmonella* and *E. coli* with zone inhibition of 10.7 mm, 18.5 mm and 16.1 mm respectively. Moreover, it was greater than gentamicin and less than ciprofloxacin activity. No activity was observed against *Staphylococcus epidermidis* while the zone inhibition for *Candida Albicans* and *Saccharomyces* were 15.9 mm and 18.9 mm respectively, which was two folders more active than Nystatin. And The antioxidant index (AI 50%) was 7.7 mg/ml after 30 minutes, while after 90 min it was 4.8 mg/ml, which indicated that the antioxidant activity of essentials oils increased with time. Thus, to achieve a good antioxidant activity, sufficient time is required.

The metals contents were determined in dried leaves by ICP-OES. Eighteen mineral elements were found in *C .incana* leaves namely; Ca, Cu, Fe, K, Mg, Mn, Mo, Na, Ni, Zn, Al, Ag, Co, Ba, Cd, and Cr. High contents of Ca (17226-22001 ppm), K (4609-17435 ppm) and Mg (2160-4100 ppm) were found. Other heavy metals were present in low quantity such as Co, Cd, Ni and Cr. Remarkable amount of Aluminum was found in six samples (513.9-1111 ppm).

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Chapter One

Introduction

1. Introduction

1.1 General

The medicinal usage of the plants is as old as humankind (Petrovska 2012). The operation of medicinal plants is based on the rich experience of healers over centuries, inherited from ancestors, healer-to-healer transfer, or developed through personal experiences over time (Khan 2014). People have relied on plants for their availability as source of food, clothing, flavors, fragrances, medicine, etc. (Gurib-Fakim 2006).

Many investigations have mentioned that medicinal plants in crude form or separated components represent the mode of medication. The plant's medicinal value is due to the presence of some chemical components that produce a physiological action on the body. Most important are the alkaloids, glycosides, essential oils, fatty oils, resins, tannins and gums, etc.

Up to nowadays, medicinal plants which formed the basis of healthcare throughout the world since the earliest days of mankind are still widely practiced in many developing countries especially in Middle East (Said, Khalil *et al*. 2002, Azaizeh, Saad *et al*. 2010).

Plants have been a source of natural medicines for the treatment of many diseases (Singh 2012). About 70-80% of the world populations, particularly in the developing countries, rely on non-conventional medicine in their primary healthcare as reported by the World Health Organization (WHO 2007).

Most of the phytogenic drugs have an advantage over synthetic drugs in having low human toxicity and in addition, huge chemical plants secondary metabolites. In particular, essential oils and herbs-derived extracts are gaining much recognition as potential source of natural and safer bioactive agents, especially against the growing microbial resistance against available chemically infective agents (Kelen and Tepe 2008).

1.2 Medicinal Plants in Palestine

Palestine is a unique region due to its diverse topographical features which lead to weather and climate changes that in turns leads to biodiversity (Mendelsohn 1999).

In Palestine, there are many medicinal plants, which are used to treat several diseases but still rarely have been described or screened in the literature. Herbal medicine is considered an integral part of the Palestinian culture and plays an indispensable role in the public healthcare. The hills and mountains of Palestine are covered with more than 2600 plant 0species of which more than 700 are noted for their medicinal benefits (Dafni, Yaniv *et al.* 1984, Said, Khalil *et al.* 2002). The efficacy, safety, toxicity, dosage and the usage instructions of medicinal plants rarely have been investigated and almost always are verbally inherited from one generation to another (Sawalha, Sweileh *et al.* 2008).

1.3 Essential oils

The fragrant mixture of liquids, obtained by hydro-distillation from aromatic plant materials, is known as an essential oil (Edwards, Hing *et al.* 2012). Essential oils are mixtures of fragrant substances or mixtures of fragrant and odorless substances. A fragrant substance is a chemically pure compound, which is volatile under normal conditions.

There were different believes regarding the role of these oils in plants. Some thought that they might help in both protecting the plant from attacks by other hostels while other thought that they may help in attracting some animals to help in pollination and seed dispersion (Packham 1997).

Calamintha genus for example contain various secondary metabolites, particularly essential oils, which contain flavonoids, triterpenoid and tannins (Monforte, Tzakou *et al.* 2011, Cavar, Vidic *et al.* 2012, Dobravalskyte, Venskutonis *et al.* 2012).

There are variations among the essential oils of *Calamintha* genus, which can depend on various factors like the geographic origin, the environmental conditions, and harvest period of the collected plant material. The environmental factors, such as temperature, relative humidity, and daylight duration, exert a direct influence on the leaves of *Calamintha Official's* (Karousou, Hanlidou *et al.* 2012, Negro, Notarnicola *et al.* 2013).

It was mention that the main components of *Calamintha* essential oils are mostly the following components: carvone, pulegone, limonene, 1,8-cineole, menthone, piperitone oxide, which might be responsible for its pharmacological activity. As was mentioned, different factors affect the yield of the essential oils and their components (Baser and Ozek 1993, Tuman, Baser *et al.* 1995, Krop, Demuth *et al.* 2012).

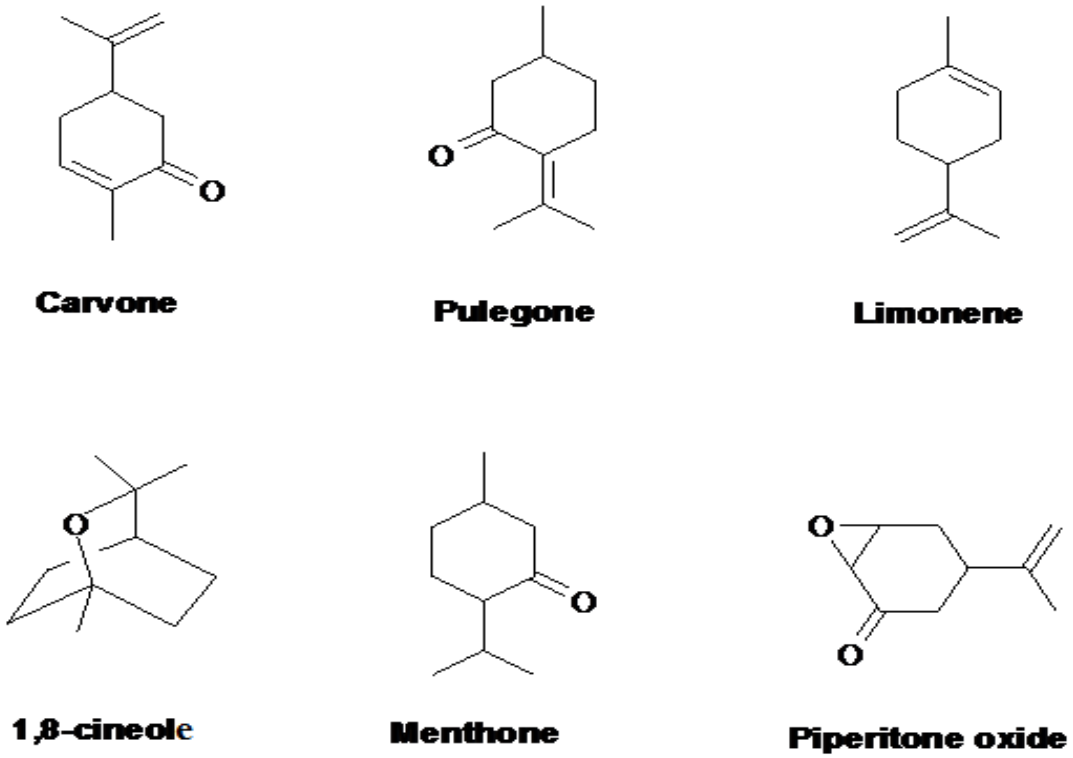


Figure 1.1: Chemical structure of main *Calamintha* essential oils components.

1.4 *Calamintha incana*

The genus *C. incana* (زعتمانة Zi'etmana in Arabic) is distributed in Europe, Mediterranean region, Central Asia, North Africa and Americas (Jung, Kim *et al.* 2003). *C. incana* plant (**Figure 1.2)** is very common in Palestine. However, up to current, in Palestine there is no single work that has been mentioned in the literature about the analysis of its components nor its pharmacological properties.

Figure 1.2: Photo of Palestinian wild *Calamintha incana* plants.

It belongs to *Lamiaceae* or *Labiatae* family. The mint family (Labiatae) has 236 genera and more than 7,000 species. It is important to humans for their flavor, fragrance, and/or medicinal properties. (Encyclopedia_Britannica 2017). Many biologically active essential oils have been isolated from various members of Labiatae family. Several species of them are used in folk medicine all around the world to treat microbial infections, cancer, malaria, inflammation, etc. (Davis and Leblebici 1982, Naghibi F., Mosaddegh M. *et al.* 2005).

1.5 Biosynthesis of *Calamintha* components

Biosynthesis of essential oils occurs through two complex natural biochemical pathways involving different enzymatic reactions. Isopentenyl diphosphate (IPP) and its isomer dimethylallyl diphosphate (DMAPP) are the universal precursors of essential oil biosynthesis. They are produced by the cytosolic enzymatic MVA (mevalonic acid) pathway or by plastidic and enzymatic 1-deoxy-d-xylolose-5-phosphate (DXP) pathway, also called the 2-C-methylerythritol-4-phosphate (MEP) pathway as in (**Figure 1.3)** (Song, Jung *et al.* 2003).

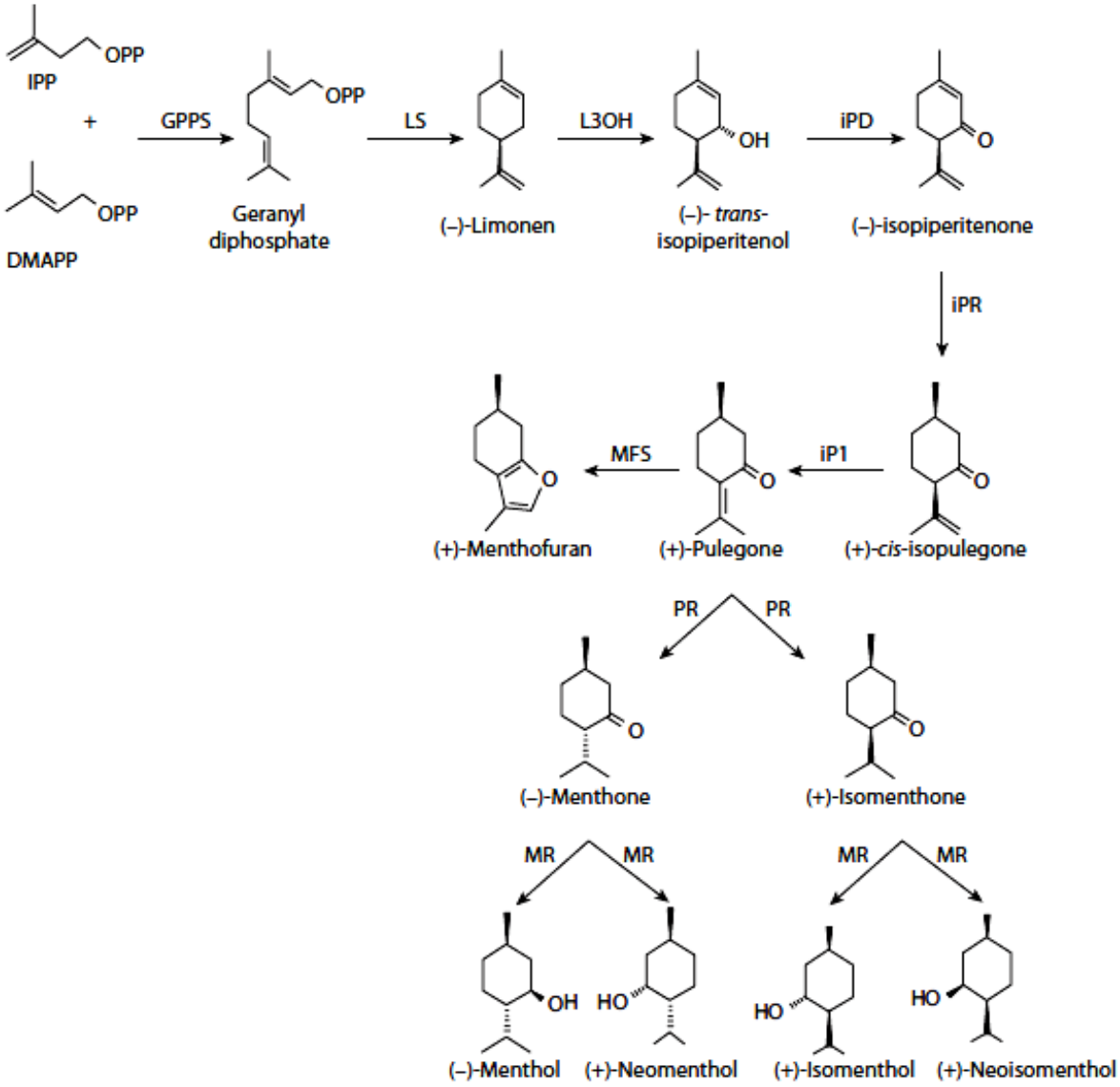


Figure 1.3: Metabolic pathway leading to the synthesis of monoterpenoids.

Pulegone is the main components of *Calamintha incana*. Which is derived from terpinolene through piperitenone. It is also the precursor of menthone, isomenthone and isopulegone as in (**Figure 1.4)** (Turner and Croteau 2004) .

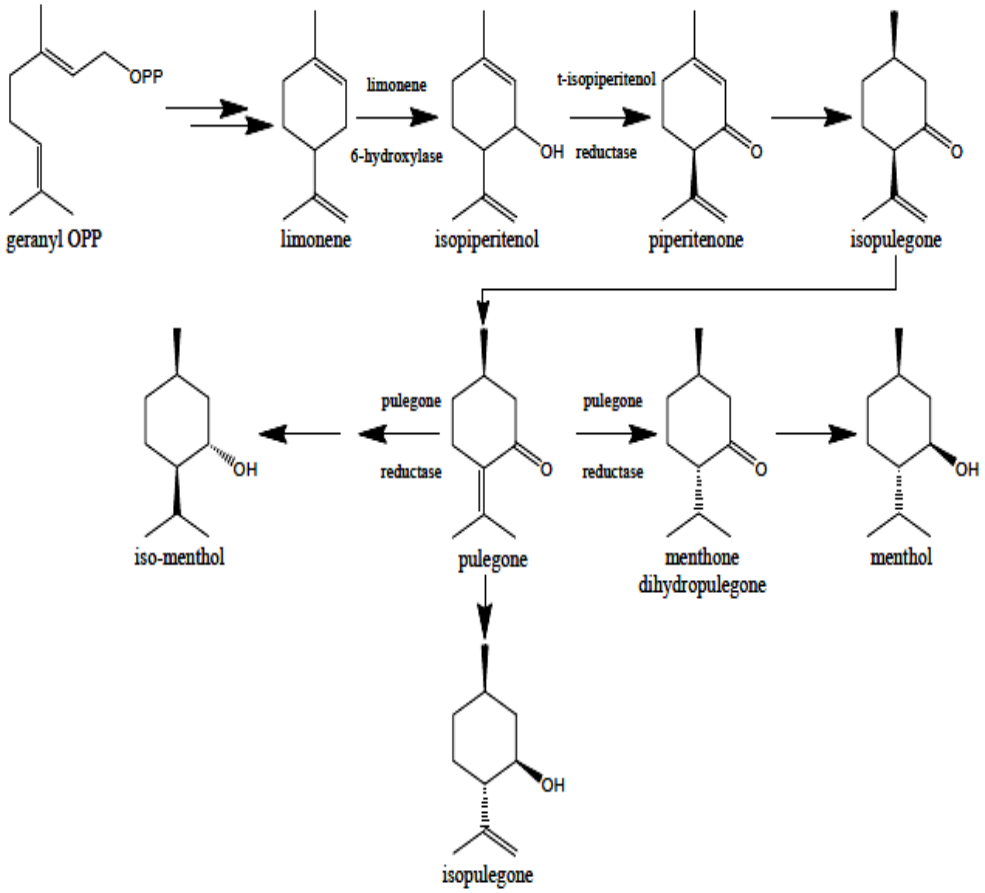


Figure 1.4: Biosynthetic pathway for pulegone and its reduced forms

1.6 Minerals in *Calamintha*

Recently, the importance of trace elements for human consumption is increased as reflected by the increased number of reports on the function of trace elements in traditional medicines. Plant minerals can be divided into macro, micro and trace elements based on needs of the metals for human body, which are required in certain amount to maintain healthy growth (Pier 1975).

As the components of enzyme related with the antioxidant effect, the importance of trace minerals i.e. Se, Mn, Cu, Zn, Fe, is being gradually emphasized with relation to their participation in maintaining normal cell metabolism, delaying of aging, and preventing cardiovascular, diabetes and immune diseases (Khan, Iqbal *et al.* 2006).

Moreover, medicinal plants might contain heavy metals as contaminants, which have negative impact on human health. The amount of metals in plants can be considered as an indication of safety and purity of plants (Li, Gao *et al.* 2002, Nasim and Dhir 2010).

To the best of our knowledge, there is no single study in the literature reporting about metal type or amount in *C. incana* species in Palestine.

1.7 Pharmacological activities of *Calamintha*

Previous studies of *Calamintha* genus have reported the presence of flavonoids, terpenoid and tannins; some of these studies indicated the presence of phenolic compounds and flavonoids which have pharmacological activities (Monforte, Tzakou *et al.* 2011, Cavar, Vidic *et al.* 2012, Dobravalskyte, Venskutonis *et al.* 2012).

1.7.1 Antimicrobial activity

Antimicrobial agents are important in reducing the global burden of infectious diseases. However, as resistant pathogens develop and spread, the effectiveness of the antibiotics is decreased. This type of bacterial resistance to the antimicrobial agents poses a very serious threat to public health, and for all kinds of antibiotics the frequencies of resistance are increasing worldwide (Al Nayem Chowdhury, Ashrafuzzaman *et al.* 2013).

Essential oils of many plants are known to have antimicrobial activity (Deans and Ritchie 1987, Cowan 1999, Marongiu, Piras *et al.* 2010). This activity could act as chemical defense against plant pathogenic diseases. Pathogens can readily penetrate at wound sites caused. Wounding of leaves, which are covered with volatile oil glands results in the rupture of glands causing the oil to flow over the wound. The existence, therefore, of antimicrobial activity in the oil, would be of considerable benefit to the plant. The antimicrobial properties of essential oils obtained from aerial parts and seeds of aromatic plants are reported (Deans and Ritchie 1987, Piccaglia, Marotti *et al.* 1993, Rasheed, Hamudi *et al.* 2010).

1.7.2 Antioxidant activity of essential oil

Free radicals or highly reactive oxygen species (ROS) are formed by exogenous chemicals or endogenous metabolic processes in the human body. These are capable of oxidizing bio-molecules such as nucleic acids, proteins, lipids and DNA and can initiate different degenerative diseases like neurological disorders, cancer, cirrhosis, atherosclerosis, arthritis etc. (Emerit and Michelson 1982, Gutteridge and Halliwell 1992, Devasagayam, Tilak *et al.* 2004).

Antioxidants are the compounds which terminate the attack of free radicals and thus reduce the risk of these disorders (Rice-Evans, Miller *et al.* 1996). Almost all organisms are protected up to some extent by free radical damage with the help of enzymes such as antioxidant compounds. Moreover antioxidant protect against the damaging effects of free radicals. Nowadays, more attention has been focused on using of natural antioxidants to protect the human.

Antioxidants play an important role in disease prevention and they are defined as compounds that inhibit or delay the oxidation of other molecules by inhibiting the initiation or propagation of oxidizing chain reactions. Their antioxidant activity is based on their ability to donate hydrogen atoms to free radicals and mainly, they are phenolic compounds with potent scavenging activity (Aruoma 1994).

The interest in phenolic antioxidants has increased remarkably in the last decade due to their great capacity to scavenge free radicals associated with various human diseases. In light of the above, it is necessary to evaluate the chemical composition of the volatile constituents obtained from *C. incana* by using SD-GC-MS analytical technique. Moreover, antioxidant activity, using different testing methods is also important (Cavar, Vidic *et al.* 2012).

1.8. Analytical methods for the analysis of essential oils

1.8.1 Methods of isolation and identification of essential oils

Steam distillation (SD) technique is usually used for extraction of temperature sensitive volatile compounds from natural products. It allows these compounds to distil and subsequently recovered at a temperature below that of the boiling points of the individual constituents. Essential oils contain substances with boiling points up to 250°C. In the presence of boiling water, these substances are volatilized at a temperature close to 100°C at atmospheric pressure. The mixture of hot vapors will allowed to pass through a cooling system condenser to form a liquid in which the oil and water comprise two distinct layers. Most essential oils are lighter than water and form the top layer. Ultimately, oil composition and constituents are determined by GC–MS (Romanik, Gilgenast *et al.* 2007).

The essential oils that identified by GC-MS. It is characterized by comparison of their mass spectra with those in the authentic NIST library and confirmed by comparison of their retention indices. And also Alkanes certified solutions can be used as reference points in the calculation of relative retention indices (RRI) (Kovats 1958).

1.8.2 Instrumentation of GC-MS

(Figure 1.5) Shows Clarus 600 GC-MS that used in this study. Brief information about its different parts and their functions are discussed below.

Figure 1.5: Perkin Elmer, Clarus 600 GC-MS used in the study.

Gas supply: Carrier gas is fed from the cylinders through the regulators and tubing to the instrument. It is usual to purify the gases to ensure high gas purity and gas supply pressure.

Injector: In the injector, the sample is volatilized and the resulting gas entrained into the carrier stream entering the GC column.

Column: GC-MS uses a gaseous mobile phase to transport sample components through columns either packed with coated silica particles or hollow capillary columns containing, the stationary phase coated onto the inner wall.

Oven: GC-MS have ovens that are temperature programmable.

Ion source: In the ion source, the products are ionized prior to analysis in the mass spectrometer. Electron impact (EI) is the major source used in GC-MS.

Mass spectrometer: MS detector connected with GC, when the compounds elute from the GC capillary column, they enter the electron ionization chamber in which, and they are bombarded with a stream of electrons causing them to break dawn into fragments. These fragments are positively charged ions with a certain mass. The mass of the fragment divided by the charge is called the mass-to-charge ratio (m/z).

Vacuum system: Mass analyzers require high levels of vacuum in order to operate in a predictable and efficient way.

Detector: The ion beam that emerges from the mass analyzer, have to be detected using electron multiplier and transformed into a usable signal. This data is then sent to a computer to be displayed and analyzed. The computer linked to the GC-MS has a library of samples to help in analyzing this data for the GC-MS is displayed in several ways. One is a total-ion chromatogram, which sums the total ion abundances in each spectrum and plots them as a function of time. Another is the mass spectrum at a particular time in the chromatogram to identify the particular component that was eluted at that time (Skoog, Holler *et al.* 2007, Hussain and Maqbool 2014).

1.8.3 Inductively coupled plasma-optical emission spectrometry

Inductively coupled plasma-optical emission spectrometry (ICP-OES) as shows in (**Figure 1.6)** is a precise, sensitive and accurate atomic emission machine that is used to determine minerals in medicinal plants.

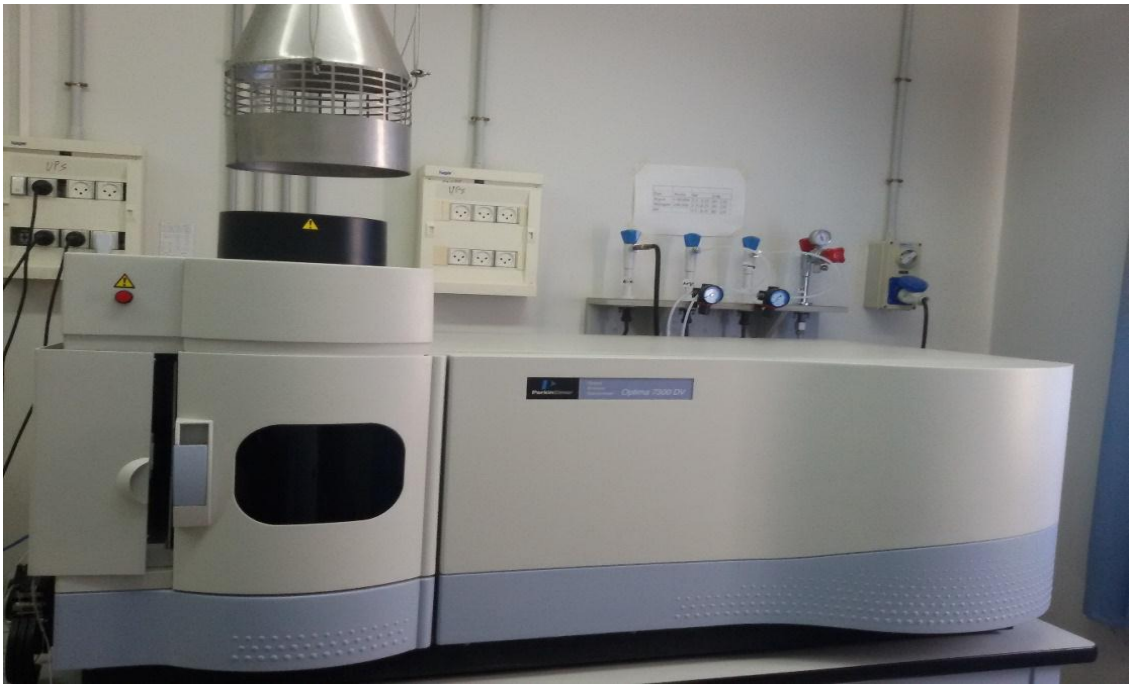


Figure 1.6: Perkin Elmer ICP-OES (DV7300) used in the study.

The sample solution is nebulized first into a radiofrequency (RF)-induced argon plasma. The tiny divided droplets of the sample are immediately dried, vaporized, and energized through collisional excitation at extremely high temperature. The atomic emission from the plasma is viewed in either a radial or an axial configuration, focused by a lens or mirror, and imaged onto the entrance slit of a certain wavelengths.

The particular wavelength excited, the monochromator, which converted it to an electrical signal by a photo detector. The signal is amplified and processed by the detector then registered and stored by the computer.

The number of photons is proportional to the concentration of the element in the sample. Up to 70 elements can be detected at single run with the combination of a polychromator and an array detector. (Fassel 1986, Boss and Fredeen 2004).

The ICP-OES technique has been applied to the analysis of a large variety of agricultural and food materials. Types of samples include soils, fertilizers, plant materials, feedstuffs, foods, animal tissues, and body fluids. Analyses of these materials are required to determine levels of essential minerals as well as levels of toxic elements in the materials. Most agricultural and food materials are generally not in the form of dilute aqueous solutions nor are they readily soluble in distilled water. Therefore, analysis of these materials by ICP-OES often requires that rigorous sample preparation procedures be carried out prior to analysis. Fortunately for the analyst, the use of modern microwave sample digestion techniques is helping to simplify the sample preparation steps for agricultural and food materials as well as many other sample types (Boss and Fredeen 2004).

1.9 Problem statement and motivation of the study

C. incana has been chosen in this research because of its medicinal reputation among elderly Palestinians. Moreover, due to the lack of information related to its secondary metabolite active components, the shortage of pharmacological and minerals contents have motivated this study.

1.10 Aim of the study

The aim of this research is to determine wild Palestinian *C. incana* volatiles and semi volatiles secondary metabolites composition by using SD-GC–MS and to evaluate some of

their pharmacological activities. Analysis will include anti-oxidant, anti-microbial and anti-fungal activities, and minerals contents of *Calamintha* dried leaves.

1.11 Objectives of the study

1. To isolate and identify the chemical composition of the essential oil from the of wild *C. incana* leaves by using SD-EI-GCMS.
2. To correlate between *C. incana's* growing location and the availability of certain compounds compositions in the plant.
3. To evaluate the antioxidant activity of the essential oil of *C. incana* using spectrophotometric method.
4. To determine the inhibitory effects of the essential oil of *C. incana* on the growth of selected bacteria and fungus in comparison with positive controls.
5. To determine the metals contents of the *C. incana* dried leaves by using ICP-OES.

Chapter Two

Literature Review

2. Literature Review

Upon extensive recent literature search, it turned out that there are very limited numbers of investigations which have been carried out on volatile constituents of *Calamintha* species at large. These studies were performed mainly in Southern European and Turkey (Marongiu, Piras *et al.* 2010, Cavar, Vidic *et al.* 2012, Conforti, Marrelli *et al.* 2012, Karousou, Hanlidou *et al.* 2012, Mancini, De Martino *et al.* 2013). These studies reported the presence of flavonoids, triterpenoid, phenolic compounds and showed antioxidant and antimicrobial activities for the plant extracts.

The composition and the antimicrobial activities of *Calamintha* species were mentioned (Monforte, Tzakou *et al.* 2011). The antimicrobial activities of the essential oils of several species of the genus *Calamintha* are partially attributed to their essential oils (Ortiz de Urbina, Martin *et al.* 1988, Nostro, Cannatelli *et al.* 2002, Nostro, Cannatelli *et al.* 2004, Krop, Demuth *et al.* 2012).

The variations among the essential oils of *Calamintha* genus studied previously may be attributed on various factors such as the geographic origin, the environmental conditions, and the harvest period of the plant material. The environmental factors, such as temperature, relative humidity, and daylight duration, exert a direct influences on the leaves were also investigated (Souleles and Argyriadou 1990, Morteza-Semnani and Akbarzadeh 2007, Radulovic and Blagojevic 2010, Karousou, Hanlidou *et al.* 2012).

Qualitative and quantitative GC-MS analysis of one of the studies revealed carvone and carveol derivatives as the main constituent in the essential oil of *Calamintha officinalis* leaves. Sixty-four components were identified, constituting 99.7% of the total oil. The major component was found to be carvone (38.7%), followed by neo-dihydrocarveol (9.9%), dihydrocarveol acetate (7.6%), dihydrocarveol (6.9%), 1,8 cineole (6.4%), cis-carvyl acetate (6.1%), and pulegone (4.1%). The essential oil showed antifungal and antimicrobial activity against Gram-positive bacteria (Monforte, Tzakou *et al.* 2011).

Nickavar and Mojabl noticed the presence of carvone (46.7%), pulegone (22.1 %), and lirnonene (24.6%) as the main components of *Calamintha officinalis* from Iran (Nickavar and Mojab 2005).

However, Bouchra *et al.* found that *Calamintha officinalis* collected in Morocco contains 1,8-cineole (36.6%) as the major component, and to a minor extent by pulegone (17.9%) and limonene (9.2%) (Bouchra, Achouri *et al*. 2003).

Morteza-Semnani and Akbarzadeh reported the presence of sesqui-terpene hydrocarbons; 1-bisabolene (9.9%), gennacrene (7.6%), 1-bourbonene (7.4%) and piperitone (5.3%), a monoterpene ketone, are the major components of the Iranian *Calaminthia officinalis* oil (Morteza-Semnani and Akbarzadeh 2007).

In another investigation, 9 species were studied in Turkey and *C. incana* was one of these species. The main components were pipertone oxide and piperitenone oxide in three samples, and pulegone, menthone, menthol and methyl acetate were found as a main components in another two samples (Baser and Ozek 1993, Baser and Kirimer 2006).

A new flavone glycoside was isolated from the aerial parts of Palestinian *C. incana* and identified based on spectral data (1-D NMR, 2-D NMR, IR, and UV) but not GCMS. However, there were no data about the composition of the essential oil, the antioxidant activity and the antimicrobial activity (Dardass, Firdous *et al*. 1999).

Composition of essential oil of *C. incana* was studied in Turkey using GC-MS, and the major components were piperitenone oxide (66.60%), limonene (6.22%) and piperitone oxide (5.91%) (Tuman, Baser *et al.* 1995).

The previously investigated findings proved remarkable qualitative and quantitative diversity in the major constituents of essential oil. The main oil components may be piperitone oxide, piperitenone oxide, pulegone, limonene, menthone, isomenthone and p-menthane compounds.

A survey among students in Palestinian universities was performed to find out the most commonly used medicinal herbs in Palestine, *C. incana* was mentioned among the most used plant (Sawalha, Sweileh *et al.* 2008).

In 2008, a study was performed to investigate the efficacy of aqueous and ethanol extracts of some Palestinian medicinal plants for potential antibacterial activity, but *C. incana* was not included (Abu Shanab 2008).

At 2013, different medicinal Palestinian plants were investigated to test their antimicrobial activity against acne inducing bacteria. However, *C. incana* was not included in the study (Ali-Shtayeh, Yaghmour *et al.* 1998, Ali-Shtayeh, Al-Assali *et al.* 2013).

Because of the increasing of health concerns and the toxicity of synthetic antioxidants, natural antioxidants have been extensively examined in recent years. The antioxidant activity of *Calamintha* species was reported by many researches. In Jordan, for example, the antioxidant activity was examined by using DPPH radical scavenging and it was comparable to ascorbic acid (EL-Agbar, Khalaf *et al.* 2008). Nonetheless, to present there is no single reference on the antioxidant activity of *C. incana* in Palestine.

Because of the increasing interest in traditional medicinal plants, it is important to determine whether they are safe for consumption and elements such as K and Na are essential for health and the quantification of these elements is important for nutritional purposes (Jia, Li *et al.* 2011).

Moreover, studies showed that the ability of different medicinal plants to accumulate minerals depends on the plant species, the geographical location and the environmental conditions and thus it is advised to examine the availability of certain minerals in plants before using them (Annan.k, Dickson. Rita A *et al*. 2013).

Many analytical methods for trace element determination in plant materials require the decomposition of the sample. Therefore, the mineralization procedure is importance for obtaining desirable results for the analytes. The wet and dry ashing procedures are slow.

In addition, these procedures are difficult to follow consistently. Microwave digestion is a rapid and efficient method for sample decomposition prior to the determination of trace metals in plants. Literature survey indicated that there is no previous studies on the *C. incana* minerals that grows wild in Palestine.

Chapter Three

Methodology

3. Methodology

3.1 Collection of plant materials

The leaves of wild *C. incana* was collected from ten different locations in Palestine between the period of June and September of 2014, except Hebron sample which was collected in August 2016. (**Table 3.1)** and (**Figure 3.1)** shows the investigated sample's locations from northern to southern Palestine.

Figure 3.1: Map showing the sites of collected *Calamintha incana* adapted from (Google Earth) 07/11/2016.

C. incana was identified and authenticated by Professor of botany Dr. Khalid Sawalha, Biology Department, Faculty of Science & Technology, Al-Quds University.

The *C. incana* leaves were air dried in the absence of light at room temperature for a about one week, and the dried samples were stored in sealed paper bags protected from light.

Table 3.1: Wild *Calamintha incana* sample's location and harvesting time.

No.	District/ Location	Harvesting Date
1	Ramallah/ Kofor Niema	19/08/2014
2	Ramallah/ Bait Rema	25/07/2014
3	Ramallah/ Bait Seera	25/07/2014
4	Jerusalem	12/06/2014
5	Jericho	12/06/2014
6	Nablus	14/08/2014
7	Tulkarm/ Anabta	10/08/2014
8	Jenin /Ya'bad	17/09/2014
9	Qalqilya/ Kofor Thelth	20/08/2014
10	Hebron	20/08/2016

3.2 *Calamintha incana* leaves extraction and GC-MS analysis

3.2.1 Reagents

GC grade n-hexane solvent and anhydrous sodium sulfate salt were purchased from Sigma-Aldrich Inc. (USA). Kovats retention index (KI) reagent which consist of alkane standard mixture were between C_{10}-C_{40} (even numbered) were purchased from Fluka, Switzerland.

All the reference standards and chemicals used in this research were kindly supplied by the Central Public Health Laboratory, Ministry of Health, Ramallah, Palestine.

3.2.2 Equipment and tools

The equipments and tools used during this study includes simple distillation system (clevenger apparatus), analytical balance (Sartorius, accuracy ±0.0001g, Germany), rotary evaporator (Steroglass-strike202, Italy), Whatman filter papers #1, separatory funnels, glass funnels, graduated cylinders, micropipetes, erlenmeyer flasks, brown glass bottels (300 and 25 ml) and brown 2ml-GC vials.

All were kindly provided by the Central Public Health Laboratory, Ministry of Health, Ramallah, Palestine.

3.2.3 Preparation of SD samples and essential oils isolation

The essential oils of the *C. incana* leaves were isolated by steam distillation (SD) using a Clevenger type apparatus by using the following this procedure:-

1- About 10 gm of the leaves from each location were grounded and mixed with 300 ml distilled water.
2- The sample was subjected to steam distillation for three hours.
3- The water distillate was extracted twice with 100 ml hexane using separator funnel.
4- The hexane fractions were combined and dried over anhydrous sodium sulfate.
5- 500 µL of hexane extract was diluted to 1 mL with hexane and 1 µL of the resulted sample was injected to the GC–MS using an optimized method.
6- The remaining hexane fractions were evaporated under vacuum by rotary evaporator.
7- The oil was collected and kept in closed amber vials at deep freezing conditions for antimicrobial and antioxidant activities examinations.

3.2.4 Instrumentations

Essential oils were tested using Perkin Elmer, Clarus 600 Gas Chromatography connected to mass spectrometer (USA). The GC-MS was operated in the electron impact ionization mode (EI) at 70 eV. Perkin Elmer autosampler was used with 2ml vials. The GC is equipped with a fused silica capillary column; DB-5 MS consisted of (5% diphenyl polysiloxane, 95% dimethyl polysiloxane) 28 m x 0.25 mm, coating film thickness is 0.25 µm (Restck, USA).

3.2.5 GC-MS chromatographic condition for steam distillation samples

Perkin Elmer EI-GC-MS was used. The flow rate of the carrier gas was 1 ml He/min. Injector temperature was set at 240oC, the source temperature was at 250oC and the interface temperature was at 260oC. Split ratio of 1:20 was adopted during the whole examination.

The column gradient temperature was held at 60°C for 2 minutes, then raised to 100°C at a ramp of 3°C/min and from 100° to 280°C at a ramp rate of 15°C/min and held there for extra 5 minutes. Solvent cut time of 5 minutes was used to eliminate the solvent peak and to normalize the response. The examined mass range was from 50 up to 500 Da, and the scan interval was 0.2 seconds.

3.2.6 Peaks identification

The identification of compounds was based mainly on matching their MS spectra with NIST mass spectral library. Moreover, Kovats Retention Index **(I)** calculation was used to confirm the identification of the results according to the following equation (Kovats 1958):

$$I = 100[n + (N - n)\frac{\log t_r^{'}(unknown) - \log t_r^{'}(n)}{\log t_r^{'}(N) - \log t_r^{'}(n)}]$$

where
n is the number of carbonatoms in the smaller alkane
N is the number of carbonatoms in the larger alkane
$t_r^{'}$ is in all cases the adjusted retentiontime (measured time minus the time of the undelayed methane or small compound.

KI values were compared with NIST values reported in the GC-MS software. Excellent agreement was obtained which indicates to the identity of the eluted peaks.

3.3 Evaluation of the anti-oxidant activity

3.3.1 Reagents

Methanol, 2,2-diphenyl-1-picrylhydrazyl (DPPH), tert-butyl-4-hydroxy toluene (BHT) were purchased from Sigma-Aldrich Inc. (USA)

3.3.2 Instruments

1. UV-Vis spectrophotometer was lambda 25 (Perkin Elmer-USA).
2. Analytical balance (Sartorius, accuracy ±0.0001g, Germany).

3.3.3 Procedure

Electrons donation ability of *C. incana* essential oils was evaluated from the bleaching of purple colored methanol solution of 2,2'-diphenyl-1-picrylhydrazyl stable radical

(DPPH) using spectrophotometric assay. After incubation period, the absorbance was measured at 517 nm.

Different concentrations of *C. incana* essential oil in methanol were prepared, 50 micro liters of each concentration were added to 2 ml of DPPH solution (DPPH concentration was 6×10^{-5} M), all samples were warped with aluminum foil and kept in dark place. And the absorbance at wavelength 517 nm was measured at three different time points, namely after 30 min, 60 min and 90 min.

At the same time tert-butyl-4-hydroxy toluene (BHT) was used as positive control, series of concentrations were prepared, 5 µL of each concentration was taken and 2 ml of DPPH was added, the final concentrations for BHT from 0.015 to 0.125 mg/ml.

Percentage of the antioxidant scavenging activity (AI %) was calculated using the equation below and then plotted against concentration to calculate the AI50 or IC50 (Piccaglia, Marotti *et al*. 1993, Brand-Williams, Cuvelier *et al*. 1995, Prakash, Brajesh *et al*. 2012).

%DPPH radical scavenging activity (AI %) = [1-(As/Ac)] X 100

Where:

- AI: Antioxidant index.
- As: Sample absorbance.
- Ac: Control absorbance.

Note:

- The positive control was BHT.
- The blank used was methanol.

3.4 Antimicrobial activity

3.4.1 Reagents

Nutrient agar (Difco), sabouraud dextrose agar (Difco) for *Candida Albicans*, purified water, 0.9 % sodium chloride AR solution, ciprofloxacin standard, gentamicin standard, nystatin standard, barium chloride AR and sulfuric acid AR were kindly supplied by the Central Public Health Laboratory, Ministry of Health, Ramallah, Palestine.

3.4.2 Instrumentation

The incubator used during this research was of B-series (bd15) with mechanical control, (Binder- Germany), while the autoclave was 3870e model (Tuttnauer- USA) and the UV-Vis spectrophotometer was lambda 25 (Perkin Elmer-USA).

3.4.3 Microbial strains and their American Type Culture Collection (ATCC)

- *Staphylococcus aureus* (25923)
- *Staphylococcus epidermidis* (12228)
- *Candida Albicans* (10231)
- *Escherichia coli* (8739)
- *Salmonella Typhimurium* (14028)
- *Saccharomyces cerevisiae (9763)*

All the above strains were obtained from Becton Dickinson, France.

3.4.4 Microbial suspension preparation

1- Each strain was suspended in 0.9% NaCl solution until reaching to 0.5 McFarland standards this was achieved by measuring transmittance by UV-Vis spectrophotometer.

2- McFarland standards were used to standardize the approximate number of microbes in the liquid suspension by visually comparing the turbidity of microbial suspension with the turbidity of 0.5 McFarland standard as in **(Figure 3.2)** and was prepared as follows:

   - 85 ml of 1% (v/v) sulfuric acid was added into a 100 volumetric flask.
   - 0.5 ml of 1.175% barium chloride solution was added drop wisely to the above flask with continuous swirling and stirring.
   - The volume was completed to 100 ml by 1% (v/v) sulfuric acid.
   - The optical density (amount of light scattered by bacteria) was measured at 625nm and should be in the range (0.08-0.1).

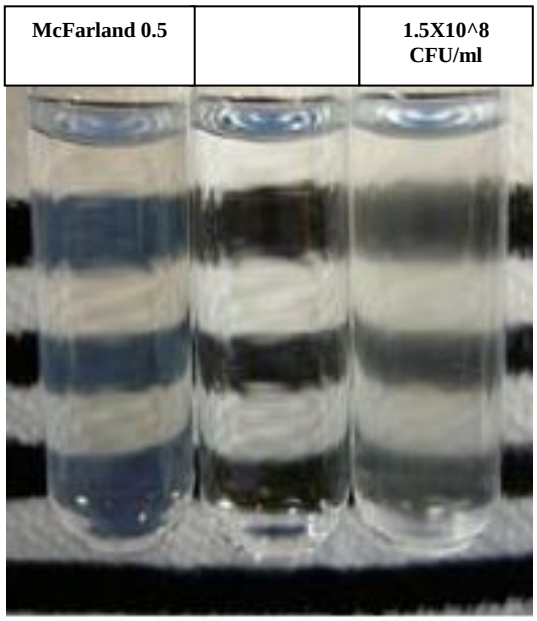


Figure 3.2: Visual comparing of microbial suspension turbidity with 0.5 McFarland standards by using Wickerham card as background.

3.4.5 Media preparation

1- One liter of nutrient agar (Difco) and sabouraud dextrose agar (Difco) was prepared in water, dissolved, boiled and sterilized at 121 °C for 20 min.
2- 20 ml of agar was added to each plate.
3- 0.1 ml of microbial NaCl suspensions was diluted with NaCl to 10 ml then 0.1 ml of the new suspension was spread with glass rod on the surface of the agar plate by streaking.
4- The final concentration of both bacteria and fungi on each plate was about 1.5×10^6 CFU/ ml.

3.4.6 Positive control preparation

Solutions of gentamicin (10 µg/ml), ciprofloxacin (10 µg/ml) and nystatin (115 IU/ml) were prepared and used as positive control.

3.4.7 Procedure

1- The test was carried out using disk diffusion method.
2- Four disks were spread on the surface of the media in each plate.
3- 5 µl of sample (*C. incana* oil) /positive control was added to each disk.

4- Each plate was incubated at 37 °C for 24 hr. for bacteria while for *Candida albicans* and *Saccharomyces cerevisiae* it was incubated at 25 °C for 72 hr.
5- Negative control for each plate type was performed by following the same procedure
6- The zone of inhibition was measured by calibrated digital caliber and the result was documented.

3.5 Minerals analysis

3.5.1 Reagents

Milli.Q ultra-pure water (Resistivity > 18 (MΩ·cm), high Purity Nitric Acid 3% optima grade was purchased from Fisher chemicals, Certified multielement standard solution 5 for ICP and Certified multielement standard solution 3 for ICP was purchased from Fluka, Switzerland. And Reference material IAEA-359 for Trace and minor elements in Cabbage from IAEA, Austria.

3.5.2 Instruments

1- Perkin Elmer ICP-OES (DV7300), USA.
2- CEM Microwave digester (MARS 6), USA.
3- Milli.Q-Millipore (Integral 10), France.

3.5.3 Procedure

All the plastic and glassware were cleaned by soaking in dilute HNO3 (1+9) and were rinsed with Milli.Q ultra-pure water and air dried before use.

3.5.3.1 Sampling

Six Samples of dried leaves of C. incana were selected from different Palestinian cities. The samples were homogenized and stored until analysis.

3.5.3.2 Digestion Procedures

The samples were prepared by microwave digestion method. Approximately 0.50 g samples of *C. incana* dried leaves and reference materials were weighed and placed into a PTFE vessels, and 10 mL HNO_3 was added to each vessel. After the digestion, the vessels were cooled to room temperature before dilution

to a final volume of 50 mL with Milli.Q ultra-pure water. The samples and blanks were prepared using the digestion parameters as in (**Table 3.2)**.

Table 3.2: Digestion Parameters for MARS 6 microwave digestion system.

Sample Type	Ramp Time (minutes)	Hold Time (minutes)	Digestion Temperature (°C)
C. incana dried leaves	20	10	200
Reference material	20	10	200
Reagent Blank	20	10	200

3.5.3.3 Blank and standard solution preparation

In order to prepare the blank and the standard solutions, 3 % HNO_3 solution was used as diluent (42 ml of High Purity HNO_3 Acid 70% diluted into 1000 ml volumetric flask with Milli.Q water used as blank and as diluent in standards preparation). All standards were prepared by using calibrated micropipette from stock multielement standard solution 5 and 3 for ICP and (**Table 3.3)** below summarized elements dilutions and concentrations.

Table 3.3: Summary of the concentrations of standards used in the ICP analysis of minerals.

Element	Stock Std. ppb	Std # 1 ppb	Std # 2 ppb	Std # 3 ppb	Std # 4 ppb
Ag	10010	10.01	50.05	100.1	1001
Al	10010	10.01	50.05	100.1	1001
Ba	10010	10.01	50.05	100.1	1001
Ca	19980	9.99	49.95	99.9	-
Cd	10010	10.01	50.05	100.1	1001
Co	10010	10.01	50.05	100.1	1001
Cr	10010	10.01	50.05	100.1	1001
Cu	10010	10.01	50.05	100.1	1001
Fe	10000	100.1	500.5	1001	10010
K	19990	1	5	10	-
Mg	40000	2	10	20	-
Mn	10010	10.01	50.05	100.1	1001
Mo	10010	10.01	50.05	100.1	1001
Na	99900	5	24.98	49.95	-
Ni	10010	10.01	50.05	100.1	1001
Pb	10010	10.01	50.05	100.1	1001
Sr	10010	10.01	50.05	100.1	1001
Zn	10010	10.01	50.05	100.1	1001

3.5.3.4 ICP-OES Analysis

Samples were cooled to room temperature and diluted to 50.0 mL with Milli.Q ultrapure water and calibration standards were prepared as mention above

The samples were run on Perkin Elmer ICP-OES (DV7300) using these analysis conditions:

- Power 1450 watt
- Plasma Gas flow : 15 L/Min
- Auxiliary Gas flow: 0.2 L/Min
- Nebulizer Gas Flow : 0.8 L /min
- Peak Algorithm : Peak Area
- Number of Replicate: 3
- Read Time: 2 -10 sec
- Plasma View: Axial and Radial

Chapter Four

Results and Discussion

4. Results and Discussion

4.1 Yield of dry leaves oils

C. incana leaves were collected from eight different Palestinian districts between June and September of 2014, except Hebron sample was collected in August 2016. The essential oils of dried leaves were then isolated using SD procedure. The harvesting time, location and the oil yield are summarized in (**Table 4.1)**.

Table 4.1: *Calamintha incana* leaves location, harvesting date and essential oils yield%

Location	Harvesting date	Average of oil yield (wt/wt)%
Ramallah/ Kifor Niema	19/08/2014	0.432
Ramallah/ Bait Seera	25/07/2014	0.398
Ramallah-Bait Rema	25/07/2014	0.451
Jerusalem/Kalandia	12/06/2014	0.367
Jericho	12/06/2014	0.384
Nablus	14/08/2014	0.329
Tulkarm/ Anabta	10/08/2014	0.389
Jenin /Ya'bad	17/09/2014	0.436
Qalqilya/ Kifor Thelth	20/08/2014	0.427
Hebron	20/08/2016	0.487

The oil yield was calculated based on weight of oil to weight of each dried sample. The average oil yield (wt/wt) was approximately 0.4%.

4.2 GC-MS analysis:

4.2.1 Identification of separated components

The *C. incana* essential oils were examined by EI-GC–MS and identified by comparing with NIST library and by calculating KI.

Seventeen major components were separated in high resolution and identified. The structure, molecular formula, retention time and KI values are summarized in (**Table 4.2)**.

The identified peaks of the *C. incana* components are depicted in the GC–MS TIC chromatogram (**Figure 4.1).**

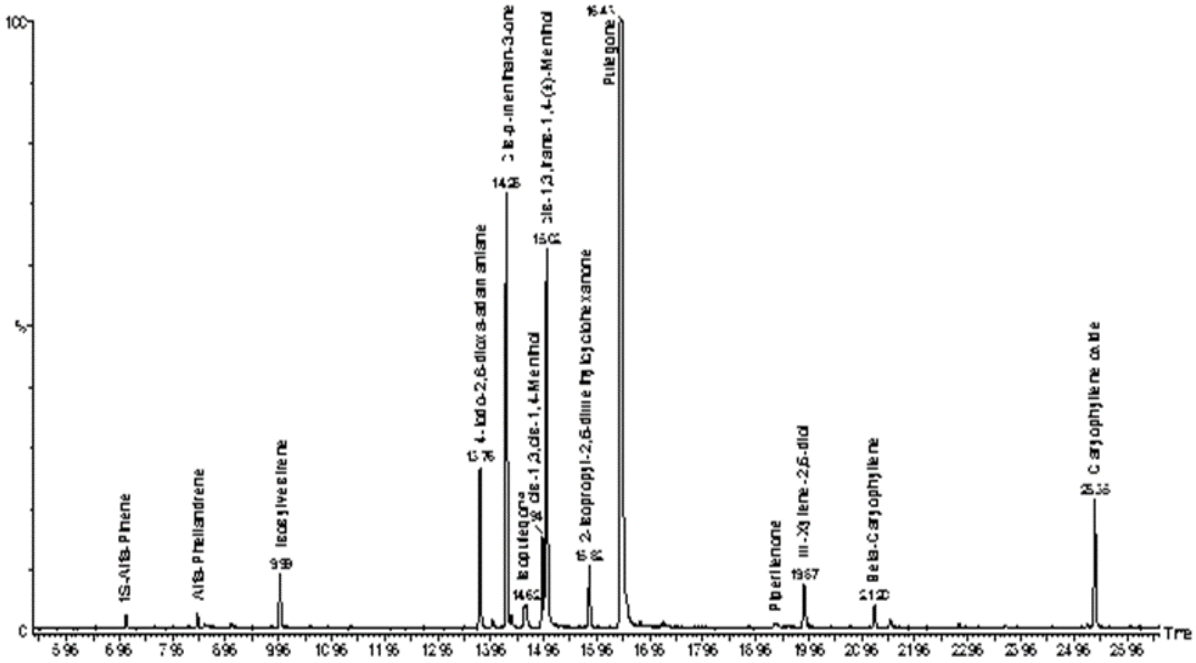
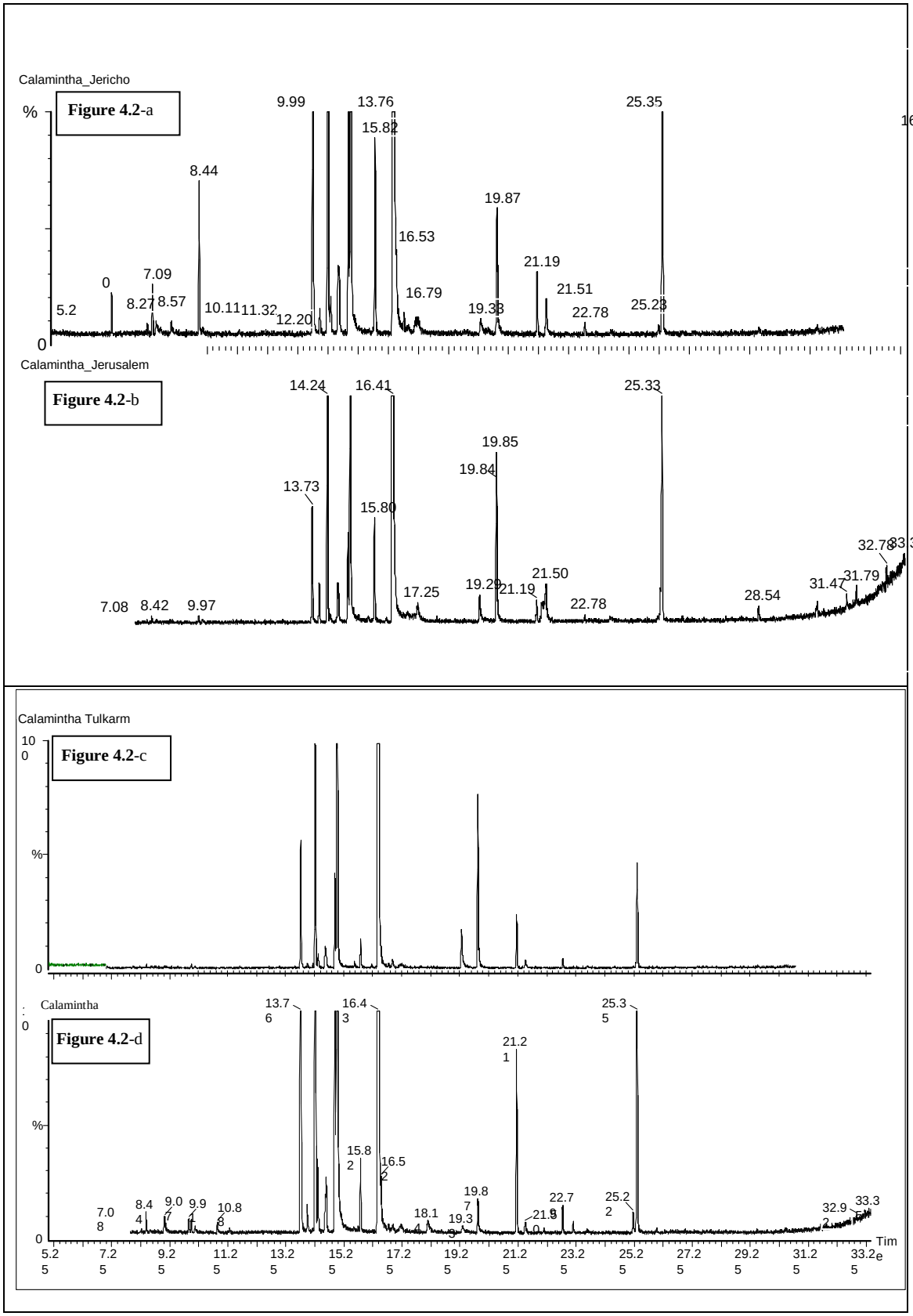
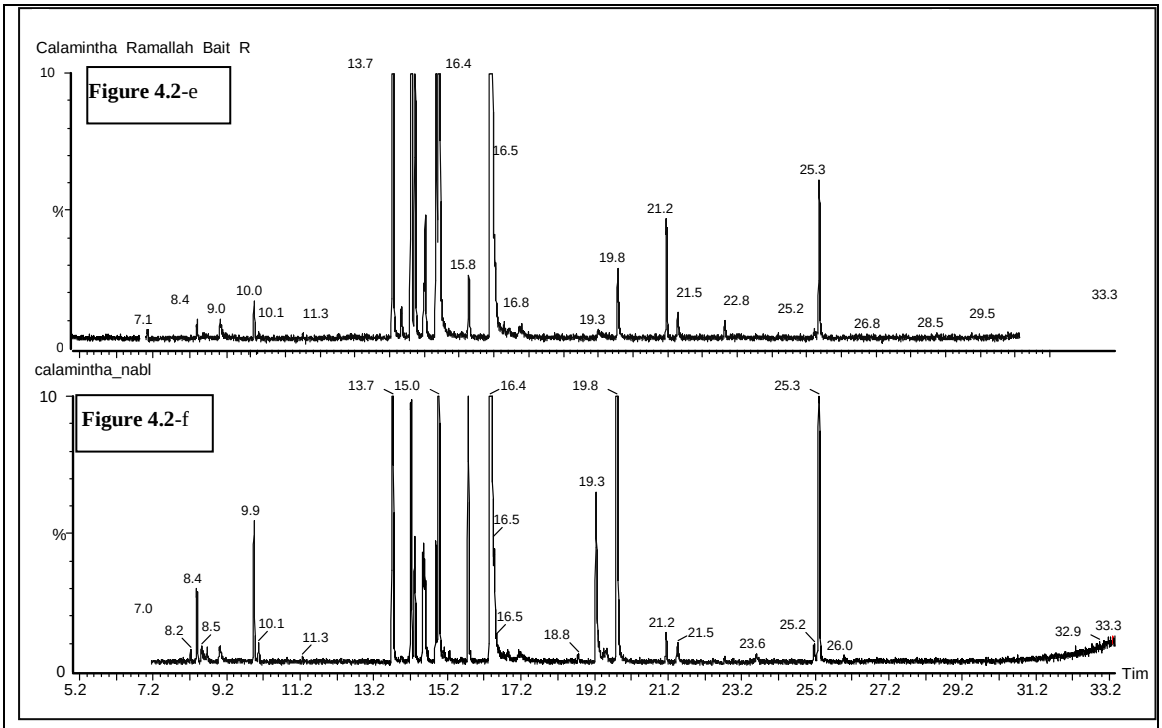
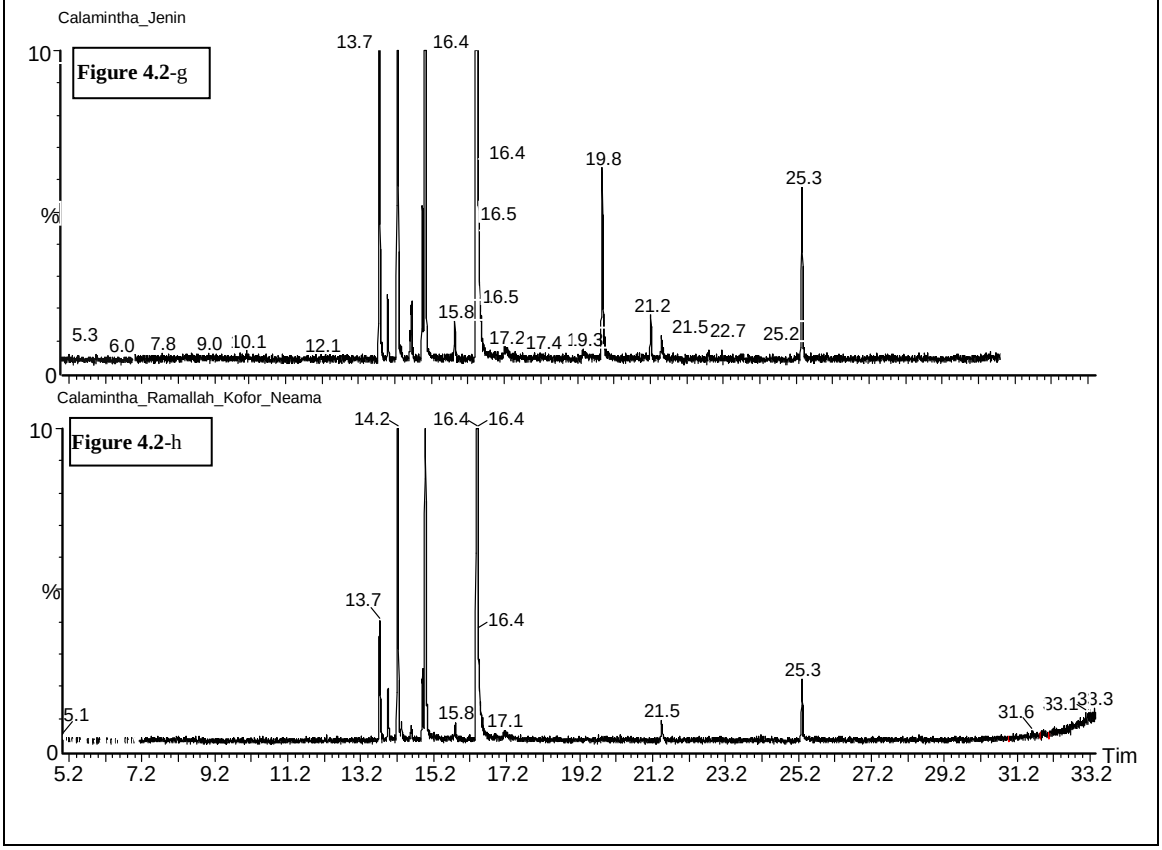


Figure 4.1: GC-MS Chromatogram (TIC) with identified peaks of the *Calamintha incana* components.

Moreover, the following are the zoomed total ion chromatograms (TIC) GCMS's that comprises some of the main components in *C. incana* samples, which were collected from different locations in Palestine as showed in **(Figure 4.2 (a –i))**.


Calamintha_Jericho
Figure 4.2-a
%
0
5.2
7.09
8.27
8.57
8.44
10.11
11.32
12.20
9.99
13.76
15.82
16.53
16.79
19.33
19.87
21.19
21.51
22.78
25.23
25.35
16.43
Calamintha_Jerusalem
Figure 4.2-b
7.08
8.42
9.97
14.24
16.41
13.73
15.80
17.25
19.84
19.85
19.29
21.19
21.50
22.78
25.33
28.54
31.47
31.79
32.78
33.36
Calamintha Tulkarm
Figure 4.2-c
10
0
%
0
Calamintha
Figure 4.2-d
0
%
0
7.08
8.44
9.07
9.91
10.83
13.76
15.82
16.43
16.52
18.14
19.33
19.87
21.21
21.90
22.75
25.25
25.35
32.92
33.35
Time
5.25
7.25
9.25
11.25
13.25
15.25
17.25
19.25
21.25
23.25
25.25
27.25
29.25
31.25
33.25


Calamintha Ramallah Bait R
Figure 4.2-e
calamintha_nabl
Figure 4.2-f
Tim


Calamintha_Jenin
Figure 4.2-g
Calamintha_Ramallah_Kofor_Neama
Figure 4.2-h
Tim

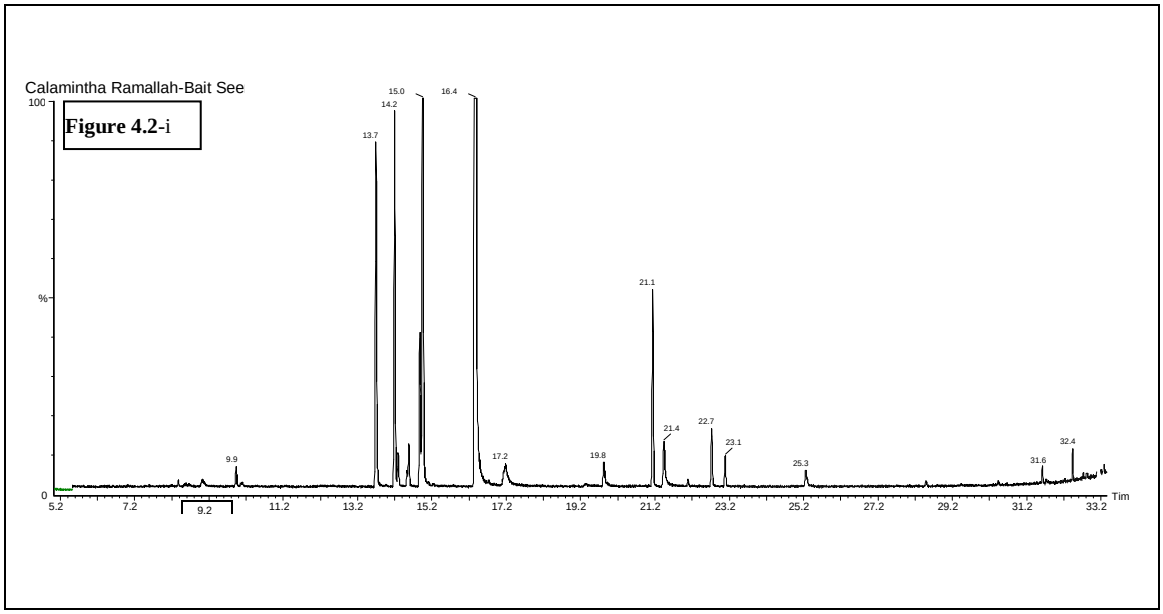


Figure 4.2: GC-MS TIC From (a –i) of *Calamintha incana* samples collected from different Palestinian locations.

Table 4.2-a: Identified components, structures, molecular formulas, retention times and KI values.

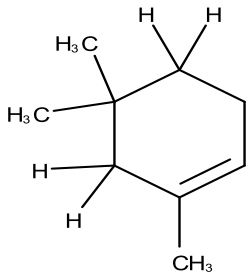
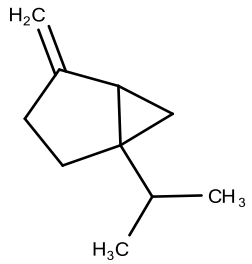
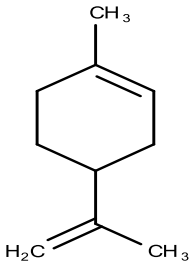
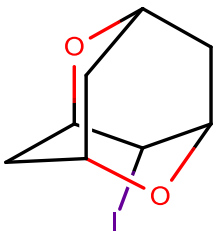
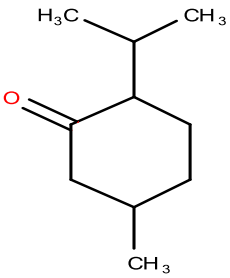
#	Component	Chemical Structure	Molecular Formula	t_R (min.)	KI value	KI Ref. value
1	1S-α-Pinene		$C_{10}H_{16}$	7.08	953	948
2	4(10)-Thujene		$C_{10}H_{16}$	8.42	1019	975
3	Limonene		$C_{10}H_{16}$	9.97	1081	1020
4	4-Iodo-2,6-dioxa-adamantane		$C_8H_{11}IO_2$	13.73	1197	1234
5	Isomenthone		$C_{10}H_{18}O$	13.97	1203	1166

Table 4.2 -b: Identified components, structures, molecular formulas, retention times and KI values.

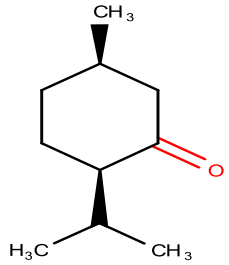
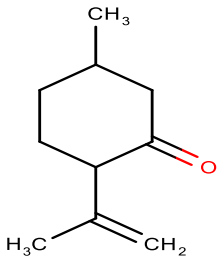
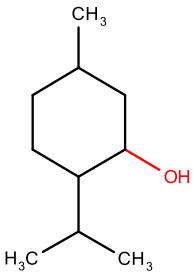
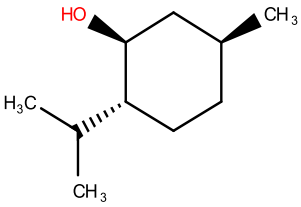
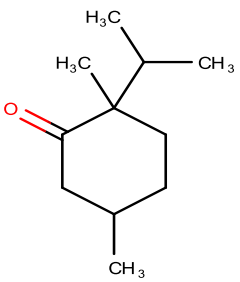
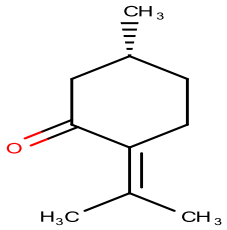
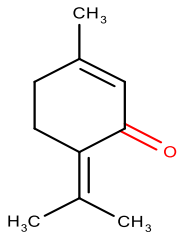
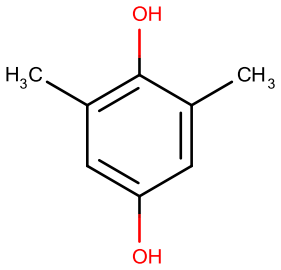
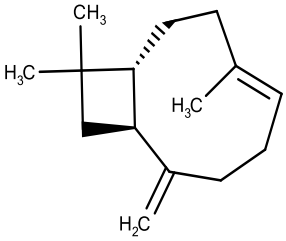
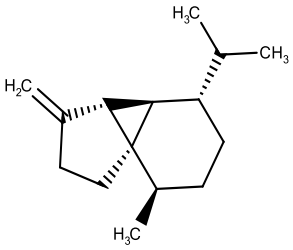
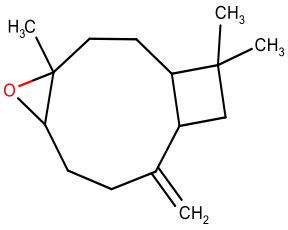
6	p-Menthan-3-one		$C_{10}H_{18}O$	14.24	1210	1151
7	Isopulegone		$C_{10}H_{16}O$	14.59	1219	1179
8	p-Menthan-3-ol		$C_{10}H_{20}O$	14.92	1227	1164
9	cis-1,3,trans-1,4-(±)- Menthol		$C_{10}H_{20}O$	14.99	1228	1164
10	2-Isopropyl-2,5-dimethylcyclohexanone		$C_{11}H_{20}O$	15.8	1247	1221

Table 4.2 -c: Identified components, structures, molecular formulas, retention times and KI values.

11	Pulegone		$C_{10}H_{16}O$	16.4	1260	1216
12	Piperitenone		$C_{10}H_{14}O$	19.29	1317	1315
13	m-Xylene-2,5-diol		$C_8H_{10}O_2$	19.85	1327	1348
14	β-Caryophyllene		$C_{15}H_{24}$	21.19	1456	1424
15	β-Cubebene		$C_{15}H_{24}$	22.77	1348	1384
16	Caryophylene oxide		$C_{15}H_{24}O$	25.33	1561	1575

The structures of *C. incana* essential oil components are presented in (**Table 4.2)**.

As showed in (**Table 4.2)**, (**Figure 4.1)** and (**Figure 4.3)**, the *C. incana* essential oil contain monoterpenoids, oxygenated monoterpenoids and to less extent sesquiterpens. Moreover, Pulegone (oxygenated monoterpenoid) was the abundant component in average concentration exceeding 50%.

All the essential oils were analyzed by GC–MS in triplicate. The relative standard deviation percentage (RSD %) were calculated for both the retention time and the peaks areas as presented in (**Table 4.3)** and **(Table 4.4)**. The RSD % values were within acceptable limits for retention time and for peak areas in particular, since in case of low concentrations (in ppm), the accepted limit usually is <15%.

Table 4.3: The RSD % of retention time (RT) for each peak area *(n=3)*

Component	RT 1	RT 2	RT 3	Average	SD	RSD%
1S-α-Pinene	7.08	7.077	7.091	7.083	0.006	0.078
4(10)-Thujene	8.42	8.419	8.448	8.429	0.016	0.195
Limonene	9.97	9.969	9.99	9.976	0.012	0.119
4-Iodo-2,6-dioxa-adamantane	13.73	13.734	13.759	13.741	0.016	0.114
Isomenthone	13.97	13.972	13.99	13.977	0.011	0.079
p-Menthan-3-one	14.24	14.236	14.258	14.245	0.012	0.082
Isopulegone	14.59	14.581	14.614	14.595	0.017	0.117
p-Menthan-3-ol	14.92	14.923	14.942	14.928	0.012	0.080
cis-1,3,trans-1,4-(±)-Menthol	14.99	14.995	15.013	14.999	0.012	0.081
2-Isopropyl-2,5-dimethylcyclohexanone	15.8	15.799	15.818	15.806	0.011	0.068
Pulegone	16.4	16.405	16.42	16.408	0.010	0.063
Piperitenone	19.29	19.287	19.308	19.295	0.011	0.059
m-Xylene-2,5-diol	19.85	19.85	19.857	19.852	0.004	0.020
β-Caryophyllene	21.19	21.189	21.2	21.193	0.006	0.029
β-Cubebene	22.77	22.781	22.777	22.776	0.005	0.024
Caryophylene oxide	25.33	25.335	25.346	25.337	0.008	0.032

The very low RSD % values of retention time advocate that the GC–MS system is reproducible.

Table 4.4: The RSD % of the peaks areas (*n=3*)

Component	Area 1	Area 2	Area 3	Average	SD	RSD%
1S-α-Pinene	26522.50	27883.00	27202.75	27202.75	680.25	2.501
4(10)-Thujene	33255.70	30195.30	31725.50	31725.50	1530.20	4.823
Limonene	113513.00	111124.50	112318.75	112318.75	1194.25	1.063
4-Iodo-2,6-dioxa-adamantane	401300.90	392757.30	397029.10	397029.10	4271.80	1.076
Isomenthone	19124.10	20086.50	19605.30	19605.30	481.20	2.454
p-Menthan-3-one	1068820.80	1024585.10	1046702.95	1046702.95	22117.85	2.113
Isopulegone	109398.30	110508.80	109953.55	109953.55	555.25	0.505
p-Menthan-3-ol	163738.10	159542.50	161640.30	161640.30	2097.80	1.298
cis-1,3,trans-1,4-(±)-Menthol	791681.70	788332.80	790007.25	790007.25	1674.45	0.212
2-Isopropyl-2,5-dimethylcyclohe-xanone	153700.20	141842.50	147771.35	147771.35	5928.85	4.012
Pulegone	6723441.00	6543603.50	6633522.25	6633522.25	89918.75	1.356
Piperitenone	12656.20	13869.40	13262.80	13262.80	606.60	4.574
m-Xylene-2,5-diol	136847.10	122538.20	129692.65	129692.65	7154.45	5.516
β-Caryophyllene	53099.20	51603.20	52351.20	52351.20	748.00	1.429
β-Cubebene	14271.5	14739.5	16244.2	15085.07	1030.75	6.83
Caryophylene oxide	308708.50	301941.90	305325.20	305325.20	3383.30	1.108

All the RSD % of peaks areas are within accepted limit <15% which indicate that even in very low concentrations, the amount of oil components determined is accurate.

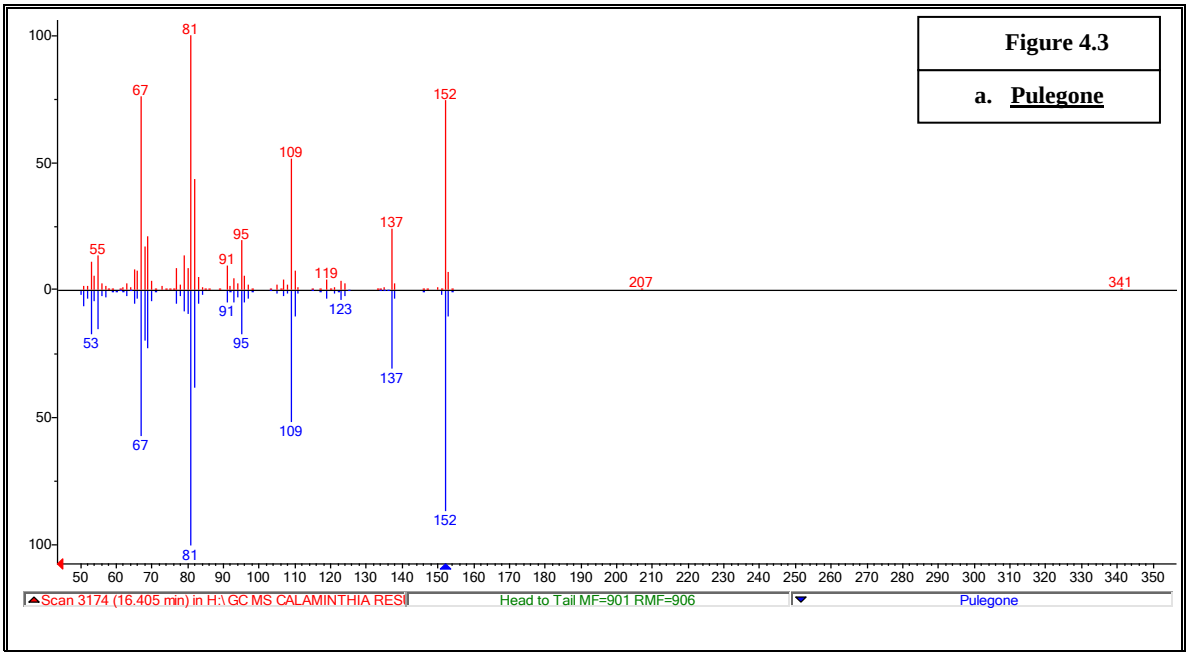
4.2.2 Interpretation of the GC-MS results

The GC–MS results were interpreted mainly based on their MS spectra and in comparison to typical NIST stored MS's. The following (**Table 4.5)** shows the MS of the major isolated volatiles present in *Calamintha incana*. It is worthwhile mentioning that each compound has some other synonyms.

Table 4.5: MS of the isolated essential oils from *Calamintha incana* and their major fragments.

#	Compound name	MW	Other m/z major fragments
1	1S-α-Pinene	136	93,92,91,77,79,41,39,121,80 and 27
2	4(10)-Thujene	136	93,41,91,77,79,39,27,69,94 and 43
3	Limonene	136	68,93,39,67,41,27,53,79,94 and 92
4	4-Iodo-2,6-dioxa-adamantane	266	139, 67, 41,93,121,39, 55,43, 95 and 79
5	Isomenthone	154	112, 69, 41, 55, 43, 139, 70, 56, 39 and 27
6	p-Menthan-3-one	154	112, 69, 41, 55, 139, 70, 97, 39, 154 and 111
7	Isopulegone	152	81,67,109, 41,152, 68, 39, 69, 82 and 53
8	p-Menthan-3-ol	156	71, 81, 95, 55, 82, 123, 41, 69, 96 and 67
9	cis-1,3,trans-1,4-(±)-Menthol	156	71, 81, 95, 55, 41, 82, 69, 123, 96 and 67
10	2-Isopropyl-2,5-dimethylcyclohexanone	168	55, 41,126, 69, 27, 43, 97, 39, 124 and 29
11	Pulegone	152	81, 67, 152, 41, 39, 109, 82, 137, 67 and 68
12	Piperitenone	150	150, 107, 135, 82, 109, 108, 91, 122, 121 and 79
13	m-Xylene-2,5-diol	138	138, 123, 137, 39, 95, 91, 109, 67, 53 and 43
14	β-Caryophyllene	204	93, 133, 91, 41, 79, 69, 105, 107, 120 and 77
15	β-Cubebene	204	161, 105, 91, 120, 41, 119, 81, 79, 93 and 55
16	Caryophylene oxide	220	43, 41, 79, 93, 91, 95, 69, 55, 67 and 81

The molecular ions and the fragmentation patterns were found to have full match with the NIST library and NIST authentic MS gave excellent conformity as shown in (**Figure 4.3)** below for *C. incana* oil components.



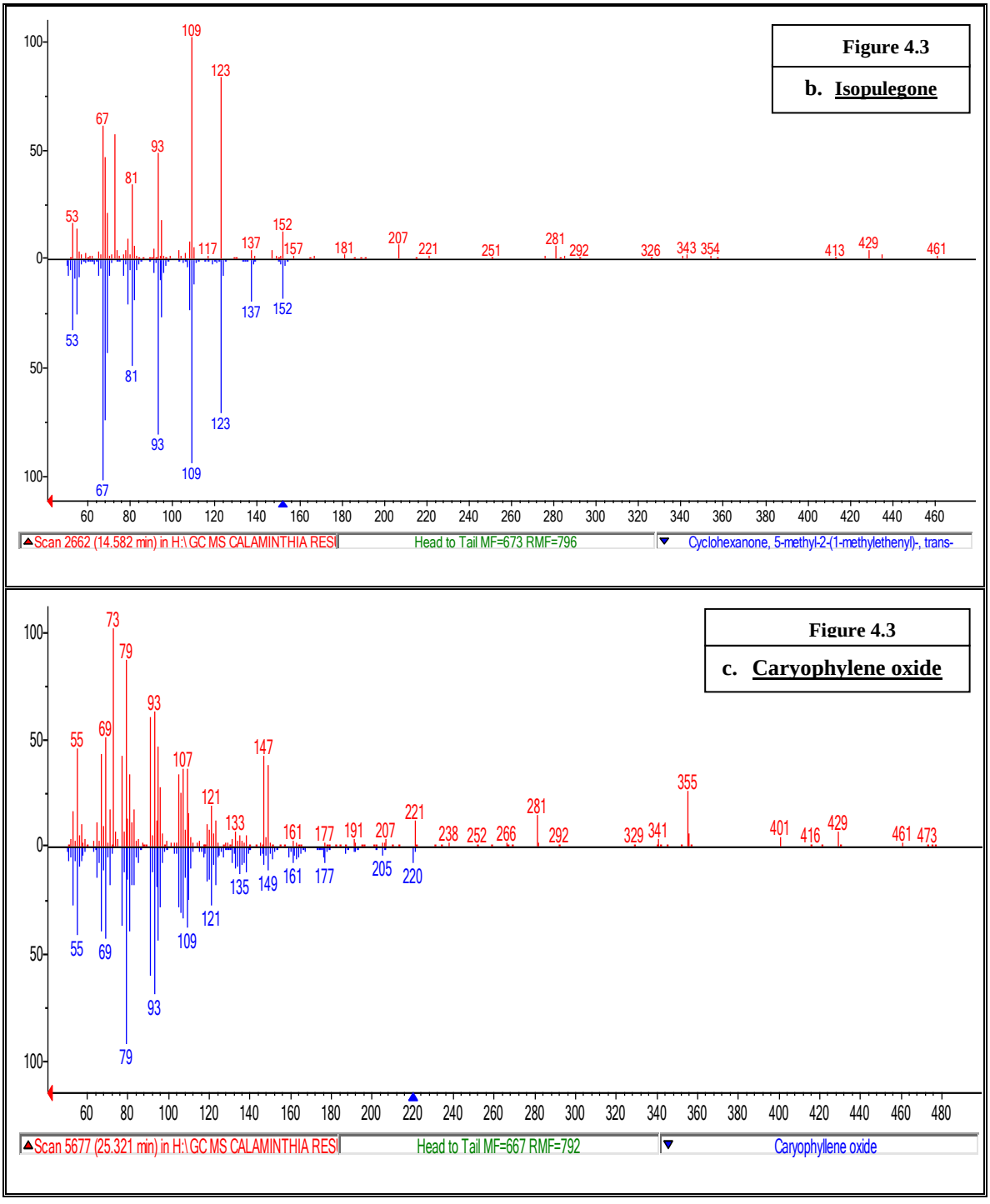


Figure 4.3
b. Isopulegone
Scan 2662 (14.582 min) in H:\ GC MS CALAMINTHIA RES
Head to Tail MF=673 RMF=796
Cyclohexanone, 5-methyl-2-(1-methylethenyl)-, trans-
Figure 4.3
c. Caryophylene oxide
Scan 5677 (25.321 min) in H:\ GC MS CALAMINTHIA RES
Head to Tail MF=667 RMF=792
Caryophyllene oxide

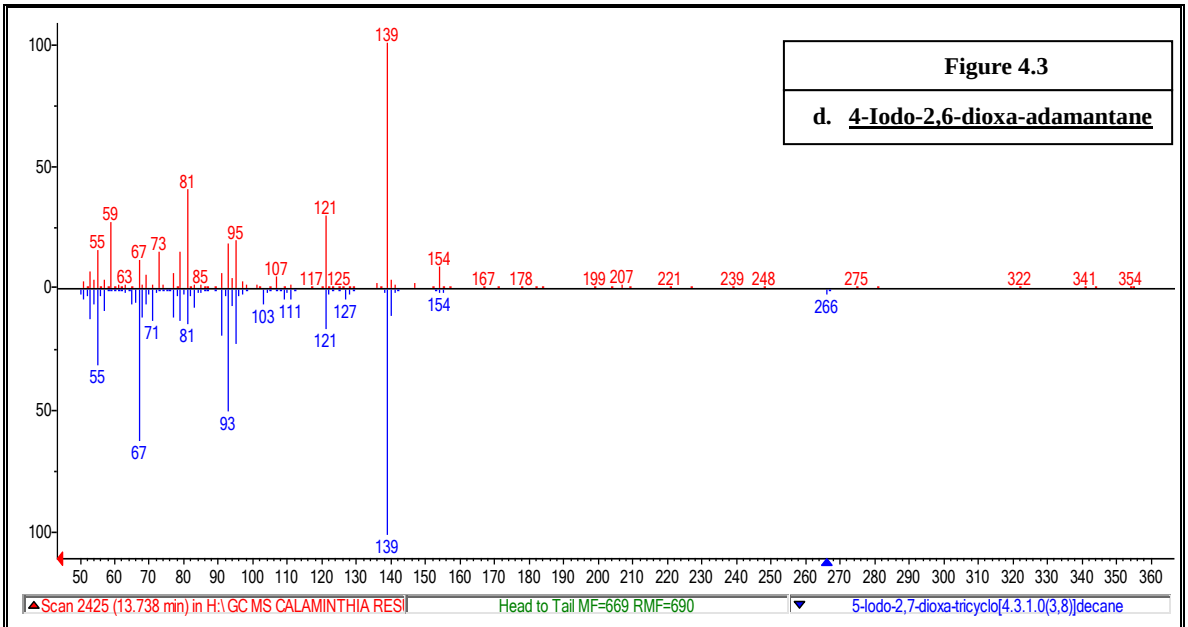


Figure 4.3
d. 4-Iodo-2,6-dioxa-adamantane
Scan 2425 (13.738 min) in H:\GC MS CALAMINTHIA RES
Head to Tail MF=669 RMF=690
5-Iodo-2,7-dioxa-tricyclo[4.3.1.0(3,8)]decane

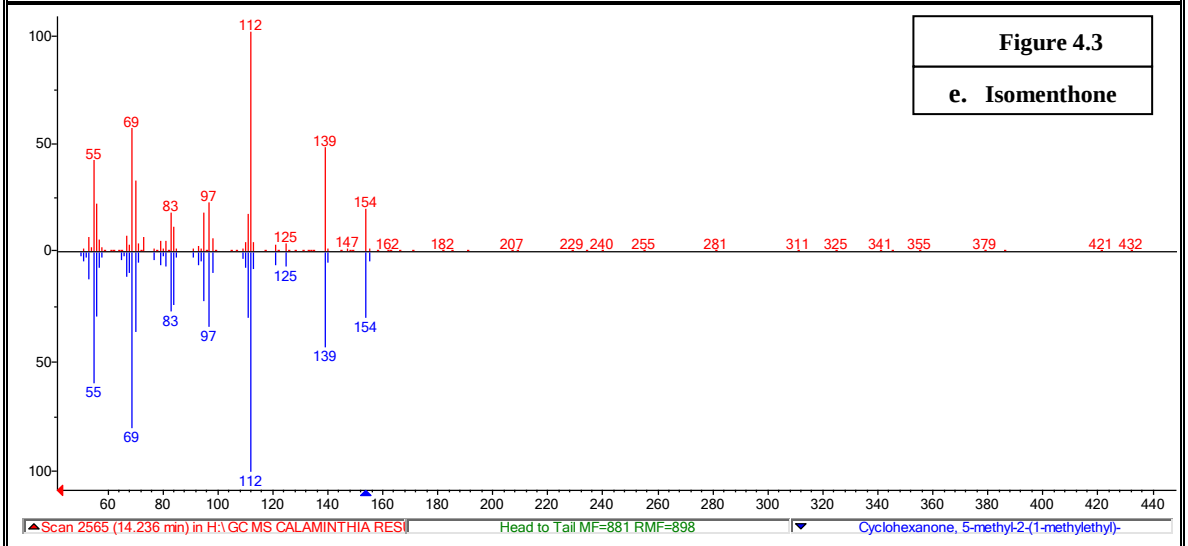


Figure 4.3
e. Isomenthone
Scan 2565 (14.236 min) in H:\GC MS CALAMINTHIA RES
Head to Tail MF=881 RMF=898
Cyclohexanone, 5-methyl-2-(1-methylethyl)-

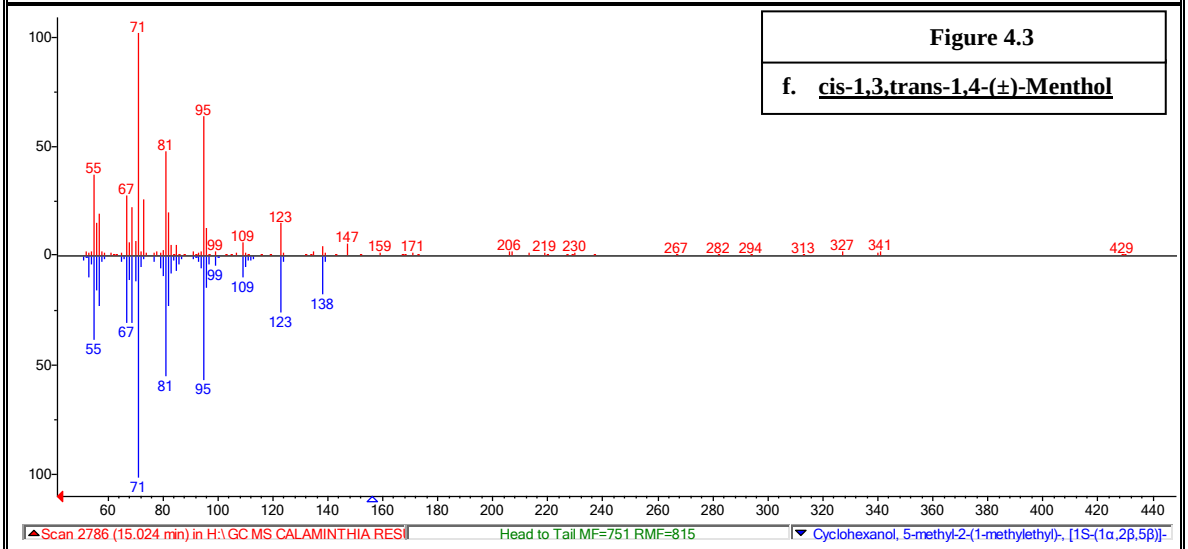


Figure 4.3
f. cis-1,3,trans-1,4-(±)-Menthol
Scan 2786 (15.024 min) in H:\GC MS CALAMINTHIA RES
Head to Tail MF=751 RMF=815
Cyclohexanol, 5-methyl-2-(1-methylethyl)-, [1S-(1α,2β,5β)]-

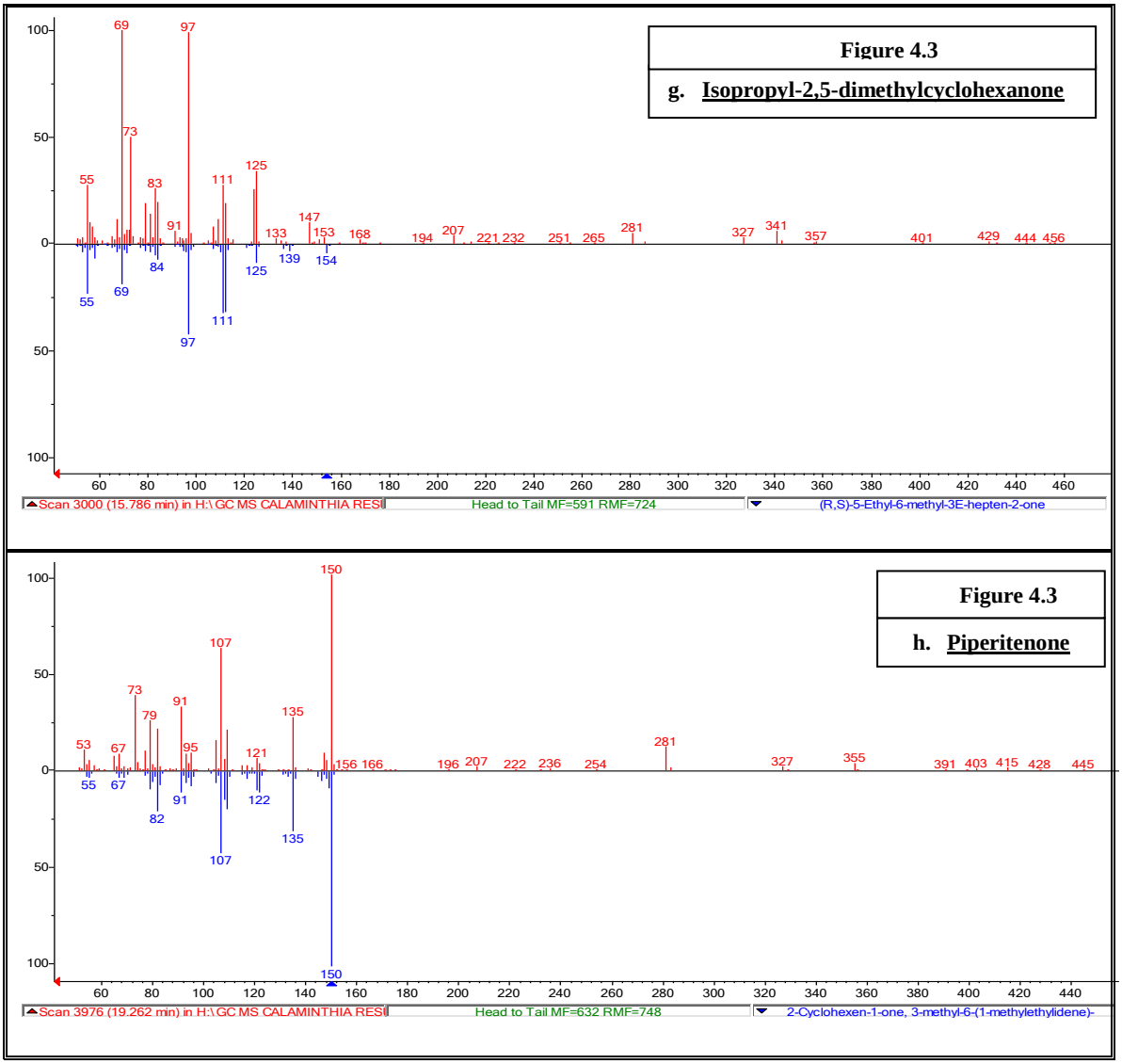


Figure 4.3: NIST MS conformation (head to tail mode) MS of selected *Calamintha incana* oil components (red) and NIST MS (blue).

4.2.2.1 *Calamintha incana* from all locations

(Figure 4.4) shows the histogram of wild *C. incana* samples from all locations. There were certain indigenous volatiles which distinguished each location by percentage of the components.

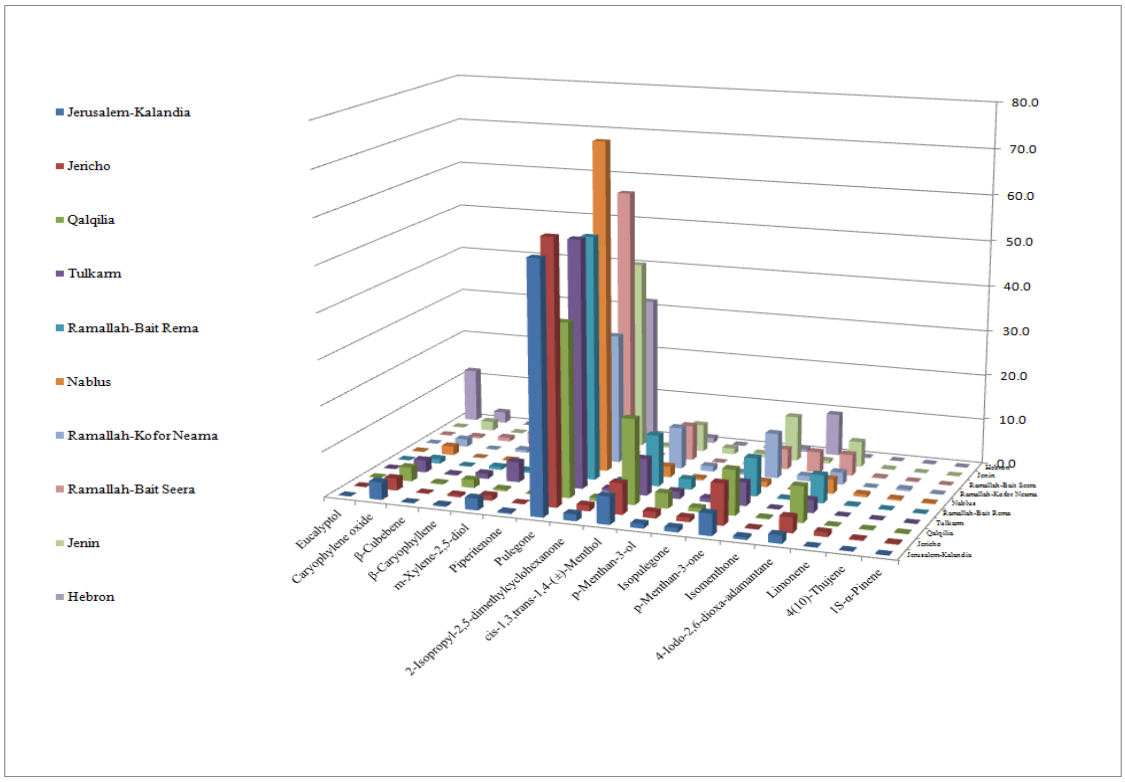


Figure 4.4: *Calamintha incana* components from all locations.

(Table 4.6) and (Figure 4.5) summarized the main components present, specifically Pulegone, p-Menthan-3-one, cis-1, 3, trans-1, 4-(±)-Menthol and Caryophylene oxide.

Table 4.6: Main components of *Calamintha incana* from all locations.

Main Component	4-Iodo-2,6-dioxa-adamantane	Iso pulegone	2-Isopropyl-2,5-dimethylcyclohexanone	Caryophylene oxide	cis-1,3,trans-1,4-(±)-Menthol	p-Menthan-3-one	Pulegone
Jerusalem Kalandia	1.81	0.95	1.51	3.88	6.09	4.78	54.55
Jericho	3.45	0.95	1.28	2.65	6.86	9.08	57.56
Qalqeelia	7.89	0.87	0.71	3.09	18.73	9.97	38.13
Tulkarm	2.80	0.84	0.65	2.71	8.08	5.15	54.26
Ramallah-Bait Rema	6.17	0.96	0.38	1.05	11.29	8.45	53.44
Nablus	3.20	0.60	1.53	1.96	2.40	1.19	72.56
Ramallah-Kofor Neama	2.68	0.43	0.30	1.69	9.18	9.95	28.64
Ramallah-Bait Seera	4.68	0.53	0.00	0.39	7.74	0.00	58.97
Jenin	5.65	0.82	0.38	2.08	6.09	9.97	41.68
Hebron	0.09	0.26	0.20	2.48	1.18	0.62	31.77

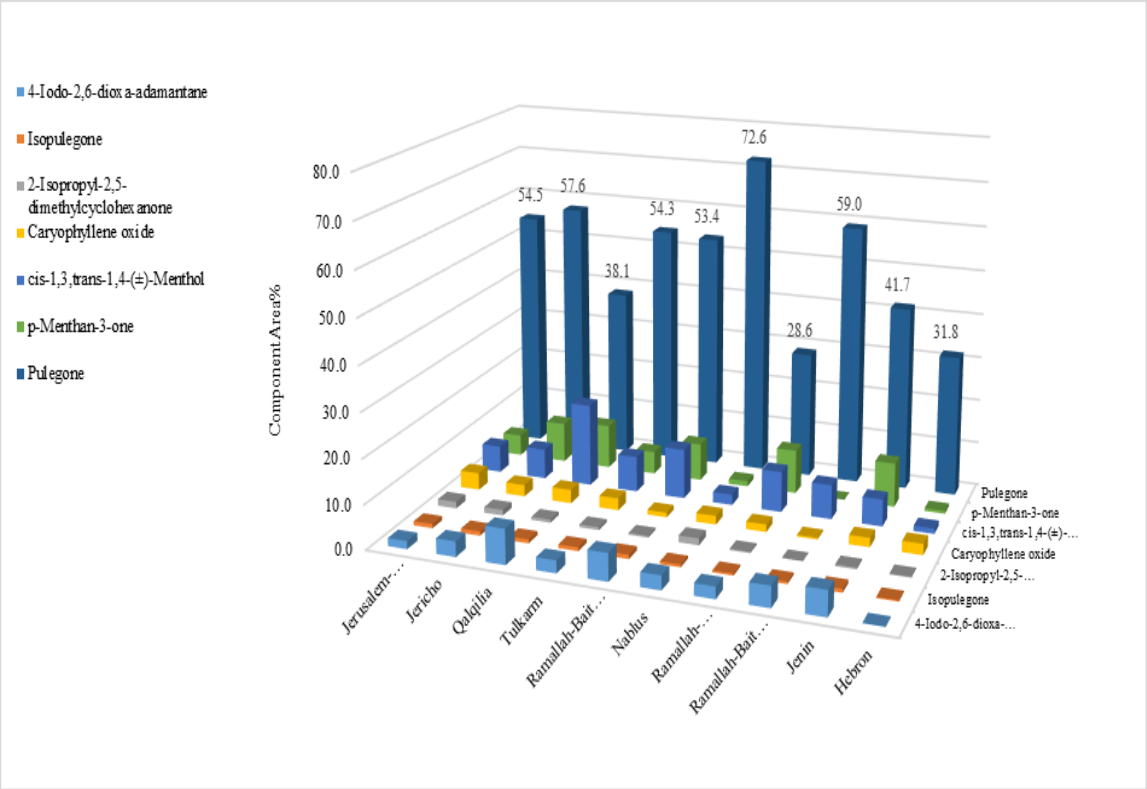


Figure 4.5: Main components of *Calamintha incana* from all locations.

Pulegone, the main component in *C. incana*, is a monoterpene ketone present in the leaves and flowering tops of several members of the mint family Lamiaceae, It is colorless oil with strong pungent aromatic mint smell. The amount of Pulegone in the various oils varies depending on several factors such as origin of the plant, yearly weather conditions, harvest date, plant age, fertilization, location and planting time (Huang, Chiu *et al.* 2003).

4.2.2.2 *Calamintha incana* from Hebron

According to the GC–MS TIC profile of *C. incana* sample from Hebron (**Figure 4.6),** only few components were observed in comparison to other samples. Moreover, Eucalyptol was found as a second abundant component (11.8%) after Pulegone (32.3%), an observation that was not seen in other samples from different locations.

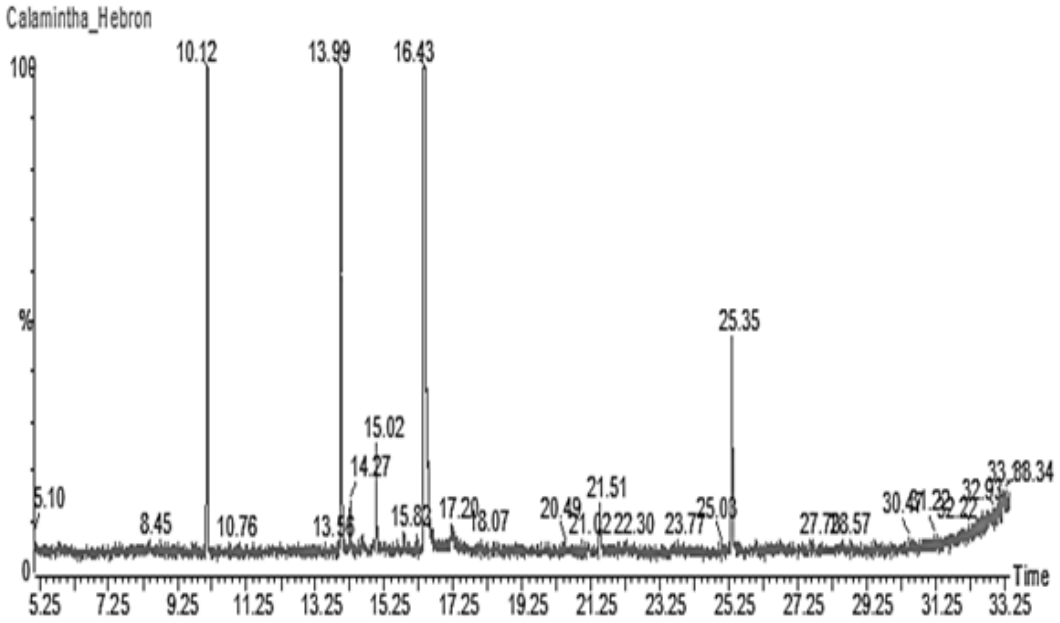


Figure 4.6: GC–MS TIC of *Calamintha incana* sample collected from Hebron.

Eucalyptol NIST MS gave excellent conformity with Eucalyptol from Hebron sample as showed in (**Figure 4.7)** and **(Table 4.7)**.

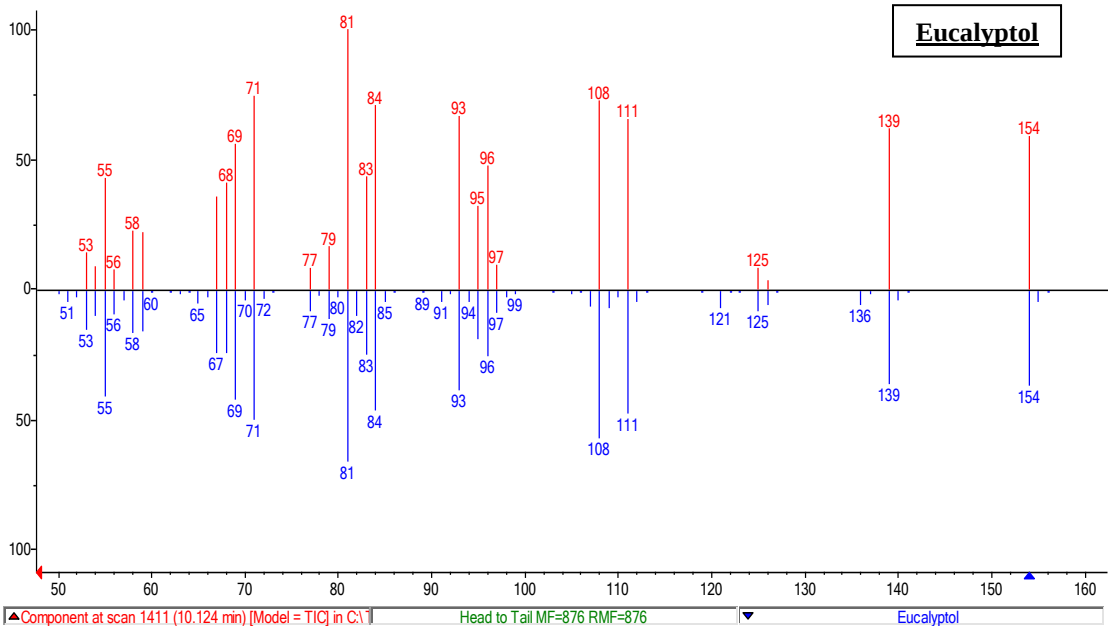
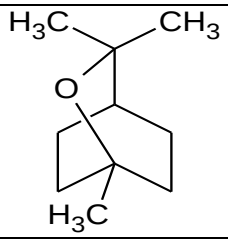


Figure 4.7: GC–MS TIC of *Calamintha incana* sample collected from Hebron NIST MS Conformation (Head to tail mode) MS of the second main *C. incana* oil component (Eucalyptol) from Hebron (red) and NIST MS (blue).

Table 4.7: Eucalyptol component of *Calamintha incana* from Hebron.

#	Component	Chemical Structure	Molecular Formula	t_R (mins)	KI value	KI Ref. value
1	Eucalyptol		C10H18O	10.1239	1059	1087

Eucalyptol is mainly used as an active ingredient in mouthwash, lozenges, ointments inhalants, body powder and cough preparations (Juergens, Stober *et al.* 1998).

In addition, it stimulates immune system response by enhancing the phagocytic ability of human monocytes (Juergens, Engelen *et al.* 2004). It has antimicrobial activity with minimal side effects either when applied topically or systemically. Because of that, it is used in many products as an active antiseptic and to reduce inflammation and pain. Due to its pleasant smell, it is used as a fragrance to impart a fresh and clean aroma in soaps, lotions, detergents and cosmetics.

4.3 Antioxidant activity of *Calamintha incana*

Antioxidants are important in many aspects especially in medical field as well as in food industry. Recently, researches on natural antioxidants from plants and food materials have been received great attention. The oxidation induced by reactive oxygen species (ROS) may cause cell damage and DNA mutation, which can further initiate or propagate the development of many diseases, such as liver injury, cancer and cardiovascular diseases (Bohm, Boeing *et al.* 1998, Liao and Yin 2000, Sanches-Silva, Costa *et al.* 2014).

The antioxidant activity of *C. incana* essential oil was examined by the DPPH method. Series of concentrations of the oil in methanol were prepared, 50 µL of each concentration were taken and 2 ml of DPPH solution (DPPH concentration $6x10^{-5}$) was added to each concentration to end up with final concentrations ranging from 0.122 to 1.35 mg/ml. All the samples were warped with aluminum foil and kept in dark place. Absorbance at wavelength 517 nm was measured at three different time intervals (30 minutes, 1 hour and 1.5 hours). At the same time, tert-butyl-4-hydroxy toluene (BHT) was used as positive control. Series

of concentrations were prepared, 5 µL of each concentration was taken and to 2 ml of DPPH was added as in *C. incana* essential oils. The final concentrations for BHT ranged from 0.015 to 0.125 mg/ml.

The antioxidant index (AI) was calculated from the following equation:

(AI %), %DPPH radical scavenging activity = [1-(As/Ac)]*100

Where:

As: Sample absorbance

Ac: Control absorbance

All AI% values were plotted against its corresponding concentration for both oil sample and the positive control as following:

4.3.1. Antioxidant activity after 30 min.

(Figure 4.8) represents the antioxidant activity of *C. incana* oil, while (**Figure 4.9)** represents the antioxidant activity of the BHT after 30 min.

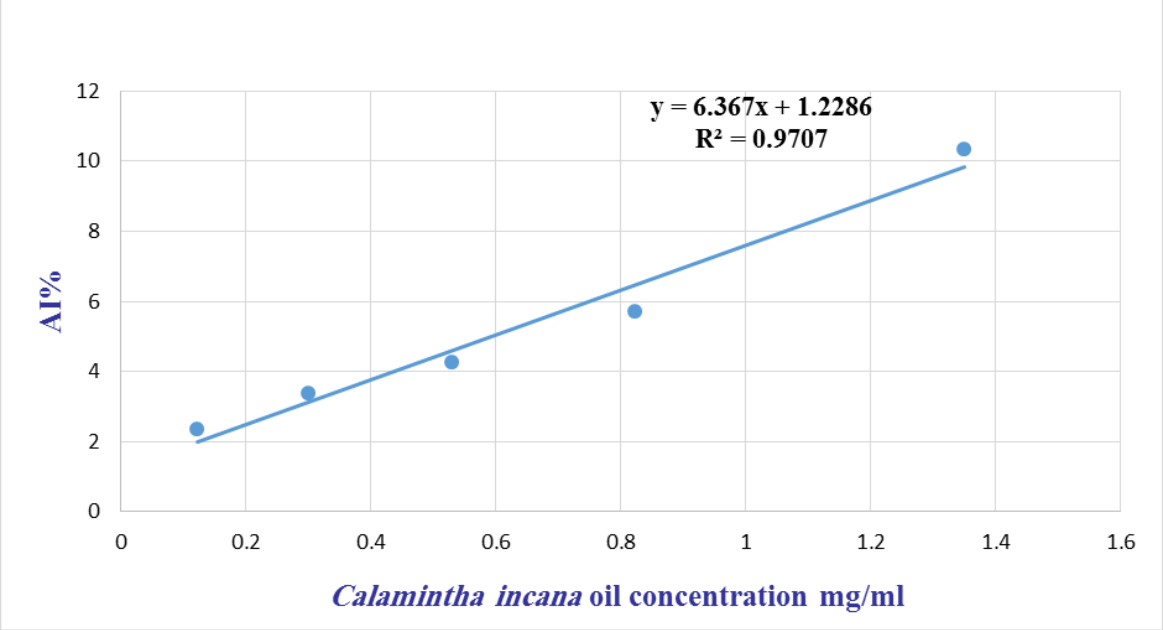


Figure 4.8: Antioxidant activity of *Calamintha incana* oil after 30 min.

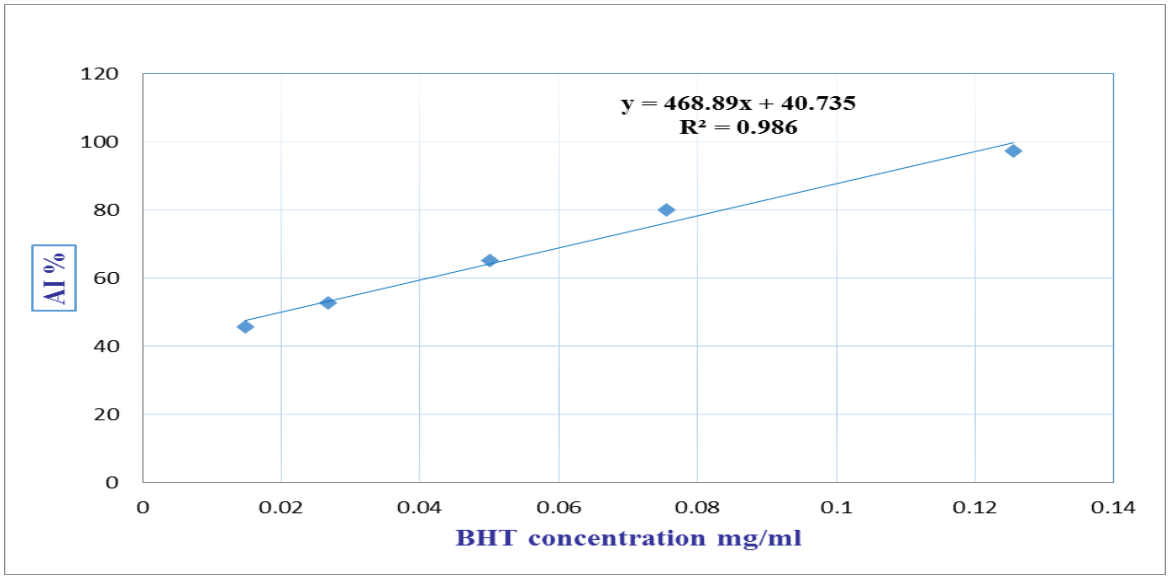


Figure 4.9: Antioxidant activity of the positive control (BHT) after 30 min.

From the (**Figure 4.8) and (Figure 4.9)**, linear relation between concentration and antioxidant activity was observed. The inhibition concentration (IC_{50}) was calculated and it was 7.7 mg/ml for the oil and 0.02 mg/ml for BHT which means that the antioxidant activity of the oil is less than that of the positive control.

4.3.2. Antioxidant activity after 60 min.

(Figure 4.10) illustrated the antioxidant activity of *Calamintha incana*, while (**Figure 4.11)** represents the antioxidant activity of the BHT after 60 min.

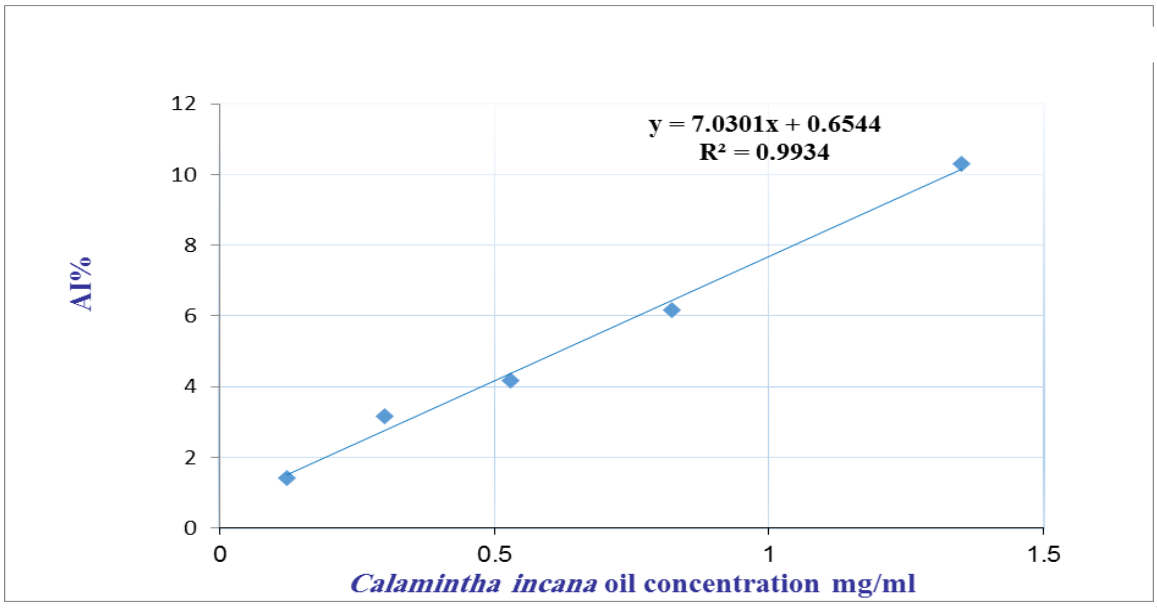


Figure 4.10: Antioxidant activity of *Calamintha incana* oil after 60 min.

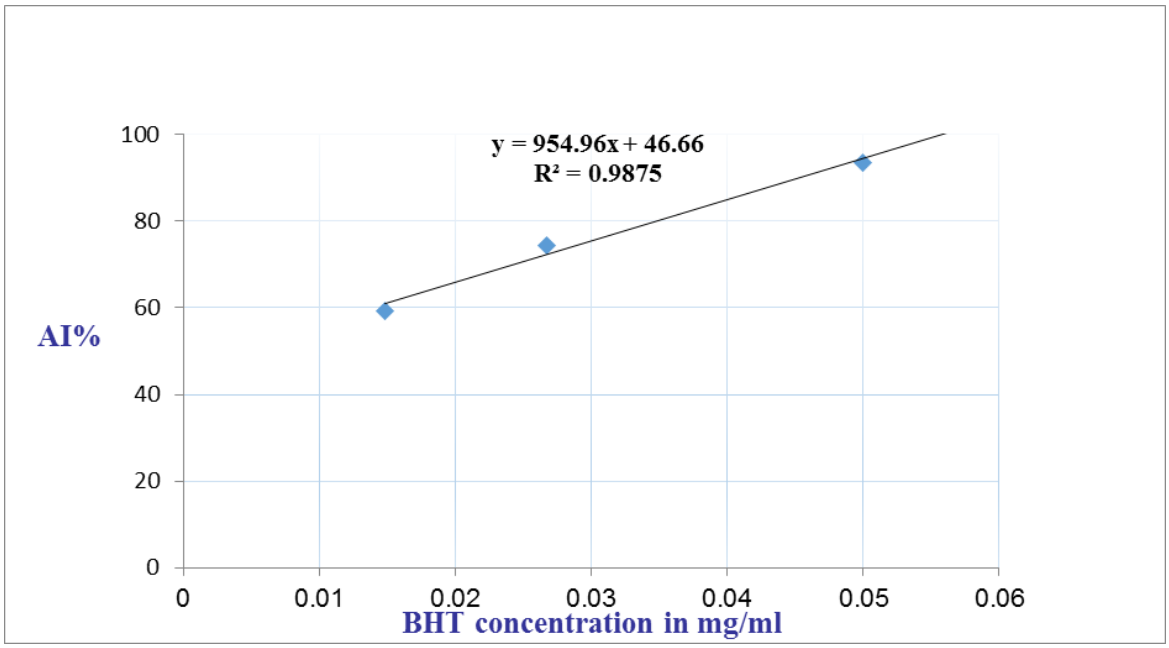


Figure 4.11: Antioxidant activity of the positive control (BHT) after 60 min.

IC_{50} after 60 min. was calculated and it was 7.02 mg/ml for *Calamintha incana*, while it was 0.004 mg/ml for BHT which means that the activity of the oil is still less than that of the positive control.

4.3.3. Antioxidant activity after 90 min.

The antioxidant activity of the oil after 90 min. is illustrated in (**Figure 4.12)** below.

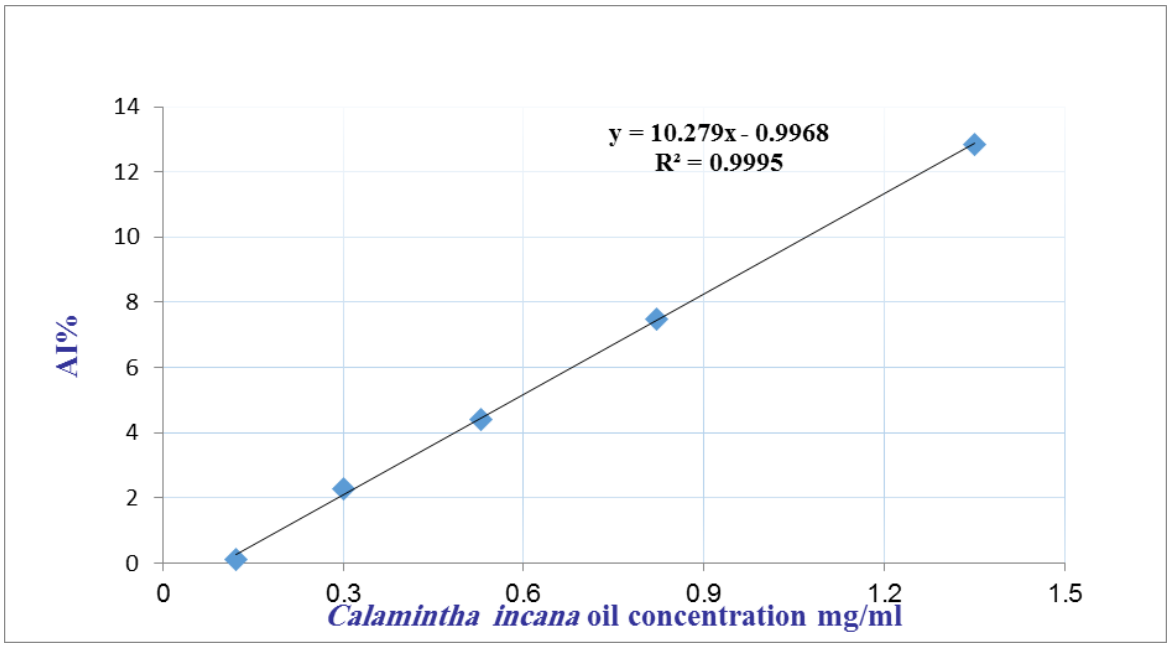


Figure 4.12: Antioxidant activity of *Calamintha incana* oil after 90 min.

IC_{50} for *C. incana* oil was 4.8 mg/ml, while it was excluded for BHT because of un-harmonized values. IC_{50} values for both sample and positive control were represented in the following histogram in (**Figure 4.13).**

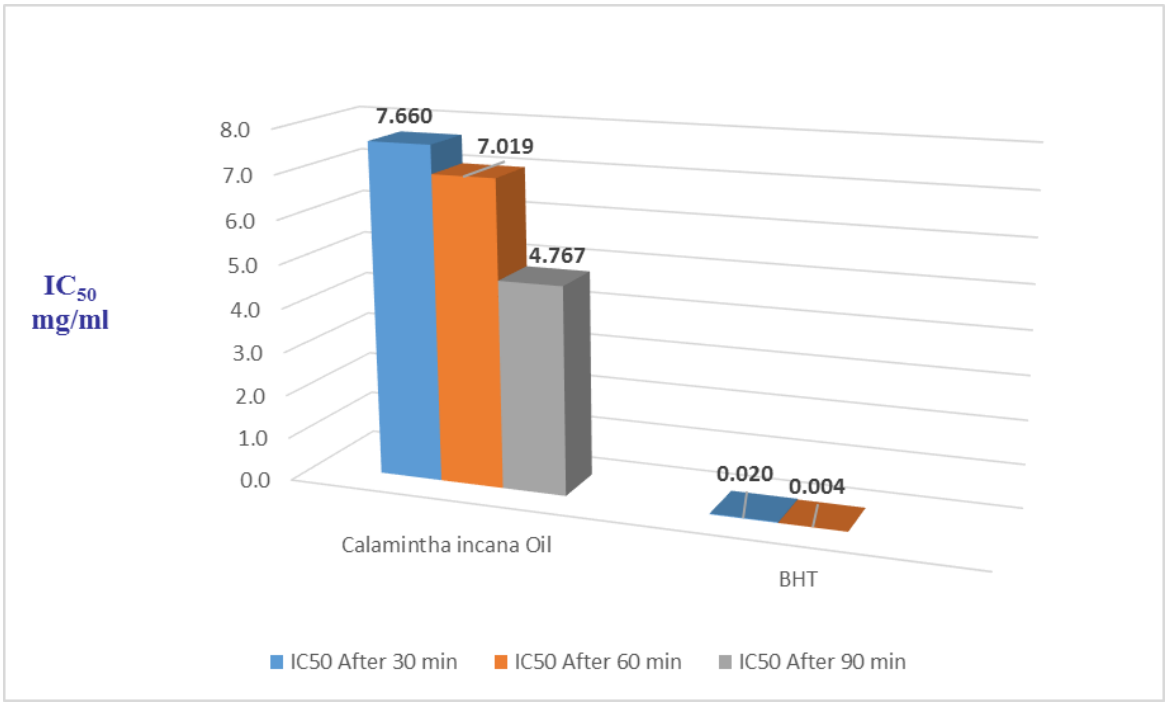


Figure 4.13: IC_{50} for both *Calamintha incana* oil and the positive control (BHT).

It is obvious from the above histogram that *C. incana* oil has antioxidant activity which is much less than that of the positive control.

Within the examined range of concentrations, free radical scavenging capacity of the tested *C. incana* oils increased in a concentration dependent manner. DPPH scavenging activities (%) were increased significantly with increasing the concentration from 0.122 to 1.35 mg/ml of *C. incana* essential oil. In addition, the activity of the oil is increased with time and to achieve a good antioxidant activity sufficient time is required.

This activity was mainly attributed to the major effective components such as oxygenated compounds and Terpenes. Preliminary GC-MS screening indicated the presence of these compounds in *Calamintha incana's* oil which are considered as free radical scavengers (EL-Agbar, Khalaf *et al.* 2008, Bozovic and Ragno 2017).

There is considerable interest in new natural antioxidants to replace the synthetic ones especially in food and cosmetic products. Many studies were conducted on the available synthetic antioxidants, BHT and butylated hydroxyanisole (BHA), to evaluate the safety of both substances. They revealed that long exposure might cause thyroid, liver, and kidney dysfunctions. Moreover, it was suggested that high doses of BHT might mimic estrogen, the main female six hormones, causing reproductive system dysfunctions (Clapp, Satterfield *et al.* 1979, Kahl and Kappus 1993).

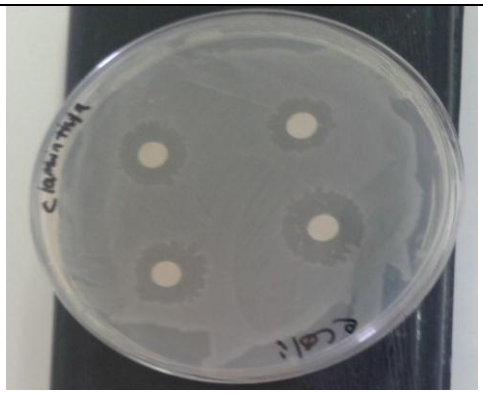
However, further research is needed to investigate the bioactivity and toxicity of *C. incana* essential oils and to test the activity of native *C. incana* at different stages of the plant life cycle and from different geographical locations.

4.4 Antimicrobial activity

In the current work, initial screening of the *C. incana* essential oil antimicrobial activity against different types of organisms was accomplished.

The antimicrobial activity of 5 µl *of C. incana* essential oil was examined on gram positive bacteria (*Staphylococcus aureus*, *Staphylococcus epidermis*), gram negative bacteria (*Salmonella typhimurium*, *E.coli*) and fungus (*Candida Albicans and Saccharomyces cerevisiae*) in the presence of positive control (gentamicin, ciprofloxacin and nystatin) by using disc diffusion method.

The zones of inhibition as in (**Figure 4.14)** were measured and the average results of zones of inhibition were summarized in (**Table 4.8) and (Table 4.9)**.



C. incana oil
antimicrobial activity against *E.coli*

C. incana oil
antimicrobial activity against *Candida*

C. incana oil
antimicrobial activity against *Salmonella*

C. incana oil
antimicrobial activity against *Staph. aureus*

Figure 4.14: Zone of inhibition of *Calamintha incana* essential oils.

Table 4.8: The antimicrobial activity results:

	Zone of inhibition (Average ± SD) mm			
	Staphylococcus aureus	***Salmonella typhimurium***	***Staphylococcus epidermis***	***E. Coli***
***C. incana* oil (5 µl)**	10.74 ±1.10	18.57±1.68	N.S	16.13±1.39
Gentamicin 10 µg/ml	8.7±0.49	9.84± 0.66	12.36±0.32	9.81± 0.73
Ciprofloxacin 10 µg/ml	N.T	21.73± 0.51	N.S	24.11±0.54
Blank	N.S	N.S	N.S	N.S

N.S: Not Sensitive.

N.T: Not tested.

Table 4.9: The antifungal activity results:

	Zone of inhibition (Average ± SD) mm	
	Candida Albicans	***Saccharomyces cerevisiae***
***C. incana* oil (5 µl)**	15.86±0.59	18.94 ±1.10
Nystatin 115 IU/ml	6.78 ±0.19	6.96 ± 0.29
Blank	N.S	N.S

N.S: Not Sensitive

Comparison between antimicrobial activities is illustrated in (**Figure 4.15)**.

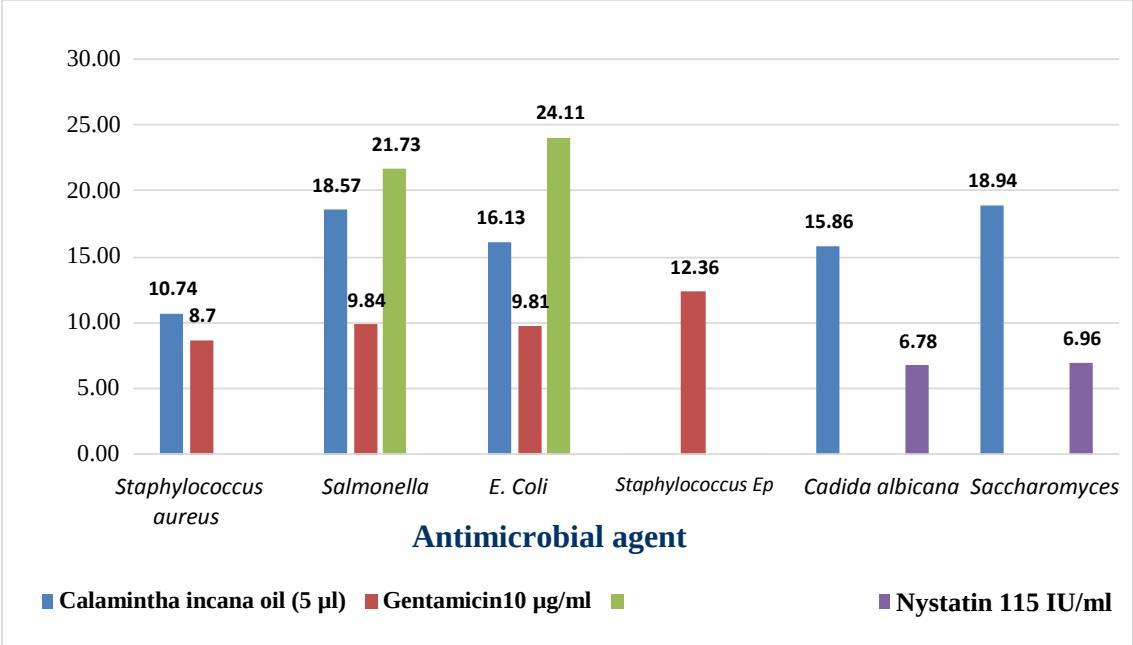


Figure 4.15: Antimicrobial activity of *Calamintha incana* oil.

The results revealed that the oil exhibited strong antibacterial activities against most of the tested fungus and bacteria. Both were sensitive, it is clear that 5 µl of *C. incana* essential oil exhibits notable antimicrobial activity against some gram positive bacteria (*Staphylococcus aureus*) and gram negative bacteria (*E.coli and salmonella*). This observed activity is superior to gentamicin in case of *Staphylococcus aureus, E.coli and Salmonella typhimurium*.

Moreover, this volume of *C. incana* oil was about three times more effective than Nystatin in case of *Candida Albicans and Saccharomyces.* Conversely, this volume of tested oil doesn't exhibit any activity on *Staphylococcus epidermis*. Although ciprofloxacin's antimicrobial activity is greater than that of 5 µl of *C. incana* essential oil against tested organism.

Regardless effectiveness, the usage of gentamicin is restricted by its toxicity. This reported toxicity remains a major problem in clinical use (Andreu, Bodet *et al.* 1985, Dulon, Aurousseau *et al.* 1988, Saleh, Abbasalizadeh *et al.* 2016). It's well known that nystatin resistance was reported after gradual exposure, seven isolates of *Candida* species became resistant (Athar and Winner 1971).

However, the antimicrobial activity of *C. incana* essential oil on *Candida* is mainly due to the antimicrobial constituents, which are capable of changing the structure and moisture of mucous membranes of fungal cells, interfering with the respiratory processes, and thus eliminate the pathogen (Chen, Zeng *et al.* 2013, Bozovic, Garzoli *et al.* 2017).

The mechanisms by which essential oil inhibit microorganisms involves different modes of action, but may be due in part to their hydrophobicity. As a result, they cause lipid partitioning of bacterial cell membranes and mitochondria, disturbing the cell structures and rendering them more permeable. Extensive leakage from bacterial cells or the exit of critical molecules and ions, will lead to death (Bozovic and Ragno 2017). According to the authors, the activity could be due to the presence of the ketones Menthone and Pulegone or Piperitone and Piperitenone with their oxides.

It was suggested that the antimicrobial activity of *Calamintha* oil was probably due to its constituents (Menthone, Pipertone oxide and Pulegone). But Pulegone has found to exhibit high antimicrobial activity (Miladinovic, Ilic *et al.* 2012).

Thus, the overall therapeutic effect of *C. incana* oil could be attributed to the synergistic interactions of individual components and to the anti-inflammatory approved effect of this oil which can lead to easier passage of the essential oils through mucous membrane. Initial screening of oil's activity revealed that this oil might be active against both bacteria and fungus which might be superior to the available antibiotics which usually act on one type only. Further studies are needed to estimate the minimum inhibitory concentration (MIC) and the safety of this oil.

4.5 Minerals analysis

4.5.1 Calibration curves

The calibration was prepared using a multi-element standard solution in a matrix of 3% HNO_3. Linear correlation coefficients for all elements were better than 0.999. (**Figure 4.16)** shows some typical elements (Ca, Mg, Na and K) calibration curves.

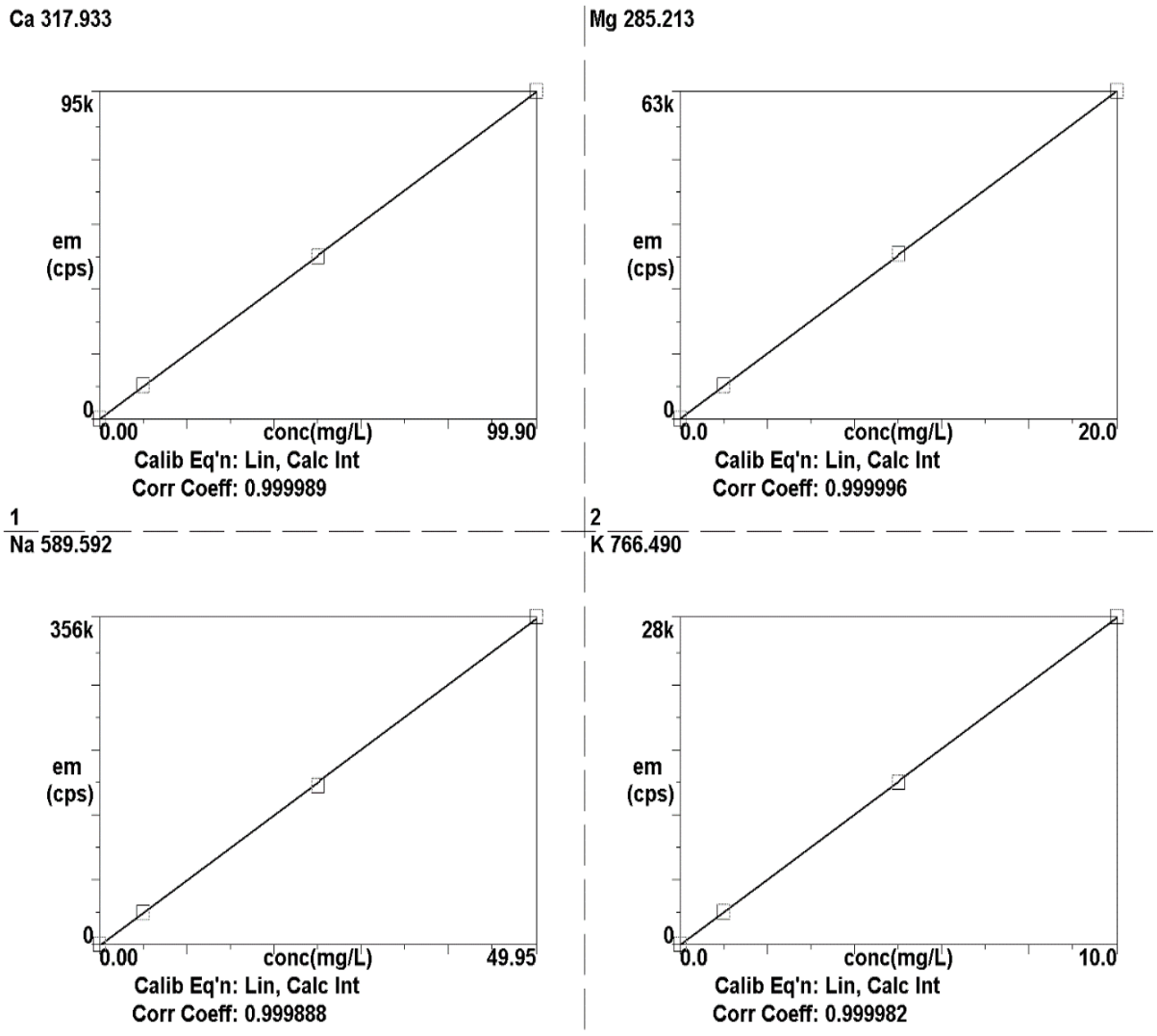


Figure 4.16: Typical element calibration curves

4.5.2 *Calamintha incana* Samples results

C. incana samples from different location were analyzed using the method described in chapter three and the results obtained from the ICP-OES are shown in (**Table 4.10)**.

Table 4.10: Minerals concentration in *Calamintha incana* leaves.

Sample ID	Element	Wavelength, nm	Conc. (Sample)	Unit	RSD (Conc.), *n=3*
***C .incana*-Jenin**	Al	396.158	557.507	mg/kg	1.63
	Ba	233.528	22.871	mg/kg	2.24
	Cd	228.802	0.361007981	mg/kg	16.94
	Cr	267.708	ND	mg/kg	-
	Co	228.613	0.63839965	mg/kg	26.26
	Cu	327.404	ND	mg/kg	-
	Mn	257.607	24.222	mg/kg	1.72
	Mo	202.032	ND	mg/kg	-
	Ni	231.601	1.949	mg/kg	7.63
	Ag	328.072	ND	mg/kg	-
	Zn	206.199	52.417	mg/kg	1.87
	Fe	238.201	503.786	mg/kg	1.61
	Na	589.604	369.837	mg/kg	0.17
	K	766.5	8986.850	mg/kg	0.42
	Ca	315.887	17226.116	mg/kg	0.21
	Mg	279.077	2161.826	mg/kg	0.63
***C .incana* - Hebron**	Al	396.158	513.868	mg/kg	1.49
	Ba	233.528	31.765	mg/kg	1.01
	Cd	228.802	ND	mg/kg	-
	Cr	267.708	ND	mg/kg	-
	Co	228.613	0.345	mg/kg	19.35
	Cu	327.404	ND	mg/kg	-
	Mn	257.607	52.713	mg/kg	1.91
	Mo	202.032	ND	mg/kg	-
	Ni	231.601	1.071	mg/kg	24.98
	Ag	328.072	ND	mg/kg	-
	Zn	206.199	58.341	mg/kg	1.38
	Fe	238.201	537.558	mg/kg	1.98
	Na	589.604	339.883	mg/kg	1.62
	K	766.5	11020.242	mg/kg	0.88
	Ca	315.887	22001.107	mg/kg	2.27
	Mg	279.077	4101.981	mg/kg	0.86
***C .incana*-Nablus**	Al	396.158	668.393	mg/kg	0.69
	Ba	233.528	29.650	mg/kg	0.46
	Cd	228.802	0.445746068	mg/kg	9.65
	Cr	267.708	ND	mg/kg	-
	Co	228.613	0.578808295	mg/kg	30.28

***C .incana*-Nablus**	Cu	327.404	ND	mg/kg	-
	Mn	257.607	21.532	mg/kg	1.75
	Mo	202.032	ND	mg/kg	-
	Ni	231.601	3.062	mg/kg	16.96
	Ag	328.072	ND	mg/kg	-
	Zn	206.199	47.874	mg/kg	1.49
	Fe	238.201	557.778	mg/kg	0.61
	Na	589.604	958.116	mg/kg	0.66
	K	766.5	4608.798	mg/kg	0.22
	Ca	315.887	21612.753	mg/kg	1.34
	Mg	279.077	2353.950	mg/kg	0.31
***C .incana*-Qalaqilya**	Al	396.158	775.978	mg/kg	0.63
	Ba	233.528	14.071	mg/kg	0.63
	Cd	228.802	0.343	mg/kg	14.55
	Cr	267.708	ND	mg/kg	-
	Co	228.613	0.826	mg/kg	2.68
	Cu	327.404	ND	mg/kg	-
	Mn	257.607	29.117	mg/kg	1.00
	Mo	202.032	ND	mg/kg	-
	Ni	231.601	1.547	mg/kg	24.69
	Ag	328.072	ND	mg/kg	-
	Zn	206.199	124.002	mg/kg	1.35
	Fe	238.201	668.820	mg/kg	1.25
	Na	589.604	378.074	mg/kg	1.51
	K	766.5	10582.683	mg/kg	1.05
	Ca	315.887	19694.044	mg/kg	1.61
	Mg	279.077	2229.473	mg/kg	0.55
***C .incana* -Ramallah**	Al	396.158	784.069	mg/kg	3.44
	Ba	233.528	13.867	mg/kg	5.54
	Cd	228.802	ND	mg/kg	-
	Cr	267.708	ND	mg/kg	-
	Co	228.613	0.643	mg/kg	12.05
	Cu	327.404	ND	mg/kg	-
	Mn	257.607	33.540	mg/kg	4.01
	Mo	202.032	ND	mg/kg	-
	Ni	231.601	2.351	mg/kg	24.26
	Ag	328.072	ND	mg/kg	-
	Zn	206.199	52.861	mg/kg	1.54
	Fe	238.201	670.348	mg/kg	3.74
	Na	589.604	662.442	mg/kg	0.82
	K	766.5	10337.254	mg/kg	0.73
	Ca	315.887	18134.988	mg/kg	0.08
	Mg	279.077	2207.490	mg/kg	0.46

***C .incana* - Tulkarm**	Al	396.158	1111.488	mg/kg	1.62
	Ba	233.528	23.538	mg/kg	1.88
	Cd	228.802	0.499	mg/kg	18.61
	Cr	267.708	ND	mg/kg	-
	Co	228.613	0.609307814	mg/kg	6.37
	Cu	327.404	ND	mg/kg	-
	Mn	257.607	47.424	mg/kg	2.84
	Mo	202.032	ND	mg/kg	-
	Ni	231.601	3.477	mg/kg	2.32
	Ag	328.072	ND	mg/kg	-
	Zn	206.199	83.928	mg/kg	1.14
	Fe	238.201	961.986	mg/kg	2.88
	Na	589.604	2516.018	mg/kg	1.71
	K	766.5	17435.242	mg/kg	1.31
	Ca	315.887	19325.803	mg/kg	0.80
	Mg	279.077	3269.818	mg/kg	1.08

Twelve elements were detected and quantified in most of dried *C. incana* leaves samples. And the concentration of not detected element was below limit of detection or RSD is more than 20%. (**Figure 4.17)** represents the availability of minerals in *C. incana* leaves.

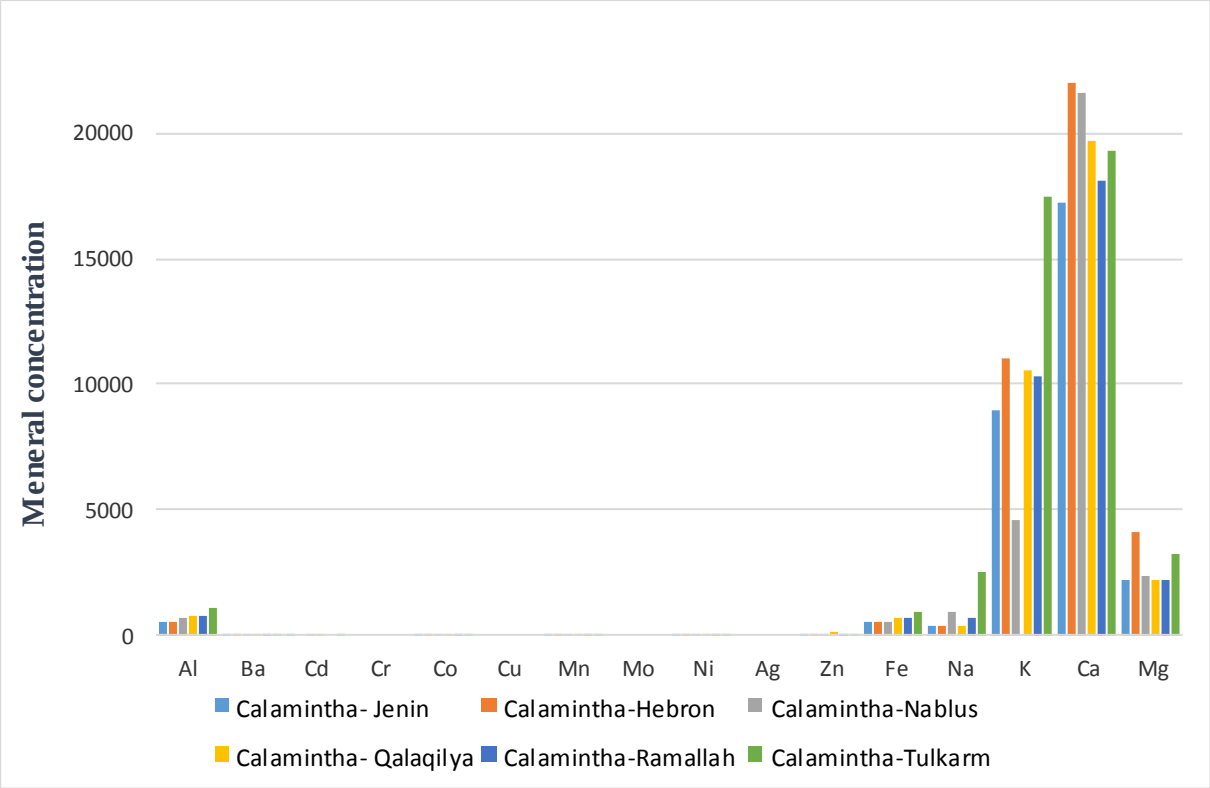


Figure 4.17: Minerals in *Calamintha incana* leaves.

The abundant minerals were Calcium, Potassium and Magnesium in the tested leaves and the high levels obtained is in consistent with its important role in biosynthesis of primary and secondary metabolic products (Ibrahim, Jaafar *et al.* 2012). The main minerals were lightened by chart in (**Figure 4.18).**

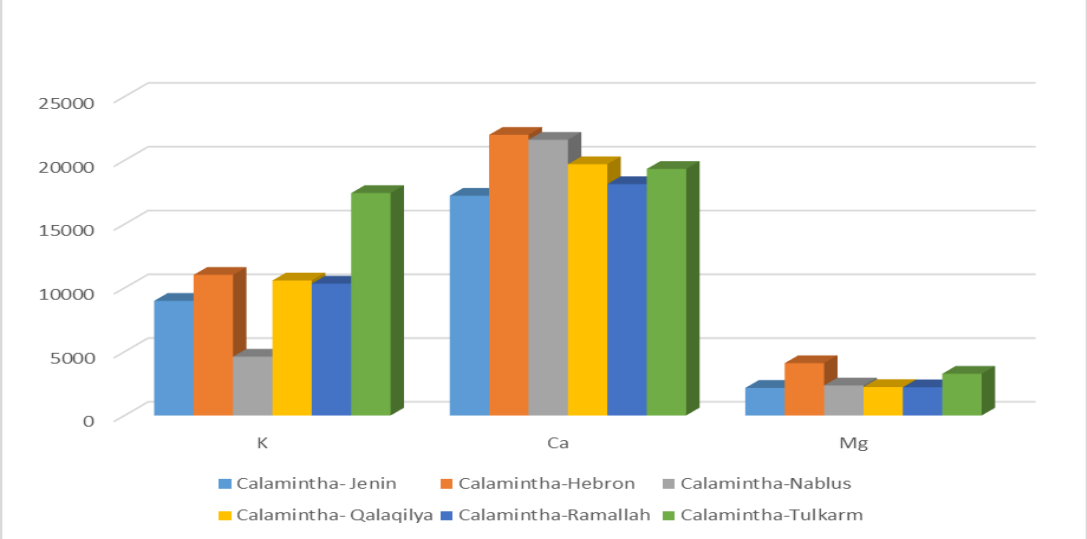


Figure 4.18: Main minerals in *Calamintha incana* leaves.

4.5.3 Limit of detection (LOD) and the limit of Quantitation (LOQ)

The blank sample was analyzed 11 times under optimized conditions, the limit of detection (LOD) and the limit of Quantitation (LOQ) were determined for each element as in (**Table 4.11)**.

Table 4.11: The detected mineral, related wavelength, LOD, LOQ and the concentration in *Calamintha incana.*

#	Element	Wavelength (nm)	LOD (ppb)	LOQ (ppb)
1	Al	396.158	16.434	54.78
2	Ba	233.528	6.489	21.63
3	Cd	228.802	6.102	20.34
4	Cr	267.708	9.297	30.99
5	Co	228.613	10278	34.26
6	Cu	327.404	16.848	56.16
7	Mn	257.607	1.899	6.33
8	Mo	202.032	17.541	58.47
9	Ni	231.601	9.078	30.26
10	Ag	328.072	6.84	22.80
11	Zn	206.199	2.379	7.93
12	Fe	238.201	17.25	57.5
13	Na	589.604	4.48	14.93
14	K	766.5	3.14	10.46
15	Ca	315.887	4.35	14.49
16	Mg	279.077	1.61	5.37

4.5.4 Reference Material Results

Reference material was analyzed by using the same conditions as *C. incana* samples and recoveries of elements were calculated as showed in (**Table 4.12).**

Table 4.12: Minerals concentration in Reference material sample.

Sample ID	Element	Wavelength	Conc. (Sample)	Unit	RSD *n=3*	Certified Value	Recovery
Reference Material	Al	396.158	117.310	mg/kg	7.79	-	-
	Ba	233.528	10.494	mg/kg	1.93	11	95.40%
	Cd	228.802	ND	mg/kg	-	0.12	-
	Cr	267.708	ND	mg/kg	-	1.3	-
	Co	228.613	0.649	mg/kg	6.25	-	-
	Cu	327.404	ND	mg/kg	-	5.67	-
	Mn	257.607	25.257	mg/kg	2.93	31.9	79.18%
	Mo	202.032	ND	mg/kg	-	-	-
	Ni	231.601	ND	mg/kg	-	1.05	-
	Ag	328.072	ND	mg/kg	-	-	-
	Zn	206.199	39.898	mg/kg	1.23	38.6	103.36%
	Fe	238.201	154.911	mg/kg	1.40	148	104.67%
	Na	589.604	454.564	mg/kg	1.36	580	78.37%
	K	766.5	24480.995	mg/kg	0.59	32500	75.33%
	Ca	315.887	14747.315	mg/kg	1.48	18500	79.72%
	Mg	279.077	1421.054	mg/kg	0.59	-	-

Due to the increasing use of medicinal plants worldwide, the safety, efficacy and quality of medicinal plants has become a major concern. There are several works that have been reported in many developed countries on minerals content of medicinal plants. Nonetheless, there is no single report regarding minerals content in *C. incana* in Palestine.

Sixteen minerals were tested namely; silver (Ag), aluminum (Al), calcium (Ca), cadmium (Cd), chromium (Cr), cobalt (Co), copper (Cu), potassium(K), sodium (Na), barium (Ba), iron (Fe), magnesium (Mg), manganese (Mn), molybdenum (Mo), nickel (Ni) and zinc (Zn).

According the USP elementals procedures at USP Method <233>, the accuracy is between 70-150% with a Relative Standard Deviation of not more than 20% (USP 2015). As shown in (**Table 4.12)** the recovers and RSD values are within the range, so the accuracy and precision results of this study was achieved.

Minerals are recognized as essential components of plants, animals and man. Therefore, elements are considered essential to life. Potassium is regarded as macronutrients in all living systems; Calcium and magnesium are required in relatively large quantities required for normal plant growth.

In *C. incana* leaves, the abundant mineral was Calcium (17226-22001 mg/kg) which is very important for life. It has an important role in building stronger, denser bones early in life and keeping bones strong and healthy later in life. Also, it has other significant roles such as in neurotransmitter release and muscle contraction. Long-term calcium deficiency can lead to rickets and osteoporosis (Ortega, Requejo *et al.* 1998). Plants could be the main source of calcium which is required daily intake for adults is nearly about 1000 mg (Tibbetts and Aub 1937, Smith, Craig *et al.* 1950, Martinez-Ferrer, Peris *et al.* 2008, Levitan, Shikany *et al.* 2013).

The second abundant mineral in the leaves was Potassium with content of (4609-17435 mg/kg). It is involved in nerve impulses, muscle contractions, including the heart muscle and in influencing osmotic balance (Stolkowski and Reinberg 1959, Dhalla, Singh *et al.* 1974, Clay and Shlesinger 1977, Bessman and Pal 1980). The optimal daily required intake (DRI) of potassium was estimated by the Institute of Medicine in 2004 as 4,700 mg of potassium. Several researches related stroke with low potassium intake (Burgess, Lewanczuk *et al.* 1999, Levine and Coull 2002, Singhal 2002, Khan, Spiers *et al.* 2013).

C. incana leaves contain relatively high concentration of potassium and low concentration of sodium which is optimal to maintain good health (Smith, Craig *et al.* 1950, Drewnowski, Maillot *et al.* 2012).

In humans, Magnesium is required in the plasma and extracellular fluid, where it helps maintain osmotic equilibrium. Lack of Mg is associated with abnormal irritability of muscle and convulsions and excess Mg with depression of the central nervous system (Indrayan, Sharma *et al.* 2005). In this study *C. incana* leaves had the Magnesium as the third abundant with range (2162-4102 mg/kg) for which the daily recommended values is about 300 mg for men and 270 mg for women.

Some of essential minerals with brief descriptions of their roles in plant, animals and human are summarized in (**Table 4.13)** bellow (Evans and Solber 1998), which were found in Palestinian *C. incana* leaves in this study.

Table 4.13-a Essential minerals with brief descriptions of their roles.

Minerals	Plants (P)	Animals (A)	Man (M)
Calcium (Ca) Macronutrient Ca, Mg, K and H cation ratio's important	Cell wall structure component. No specific recommendation other than liming low pH soils, 5.5 or less.	The 5th most abundant body mineral.	The primary structural bone mineral.
Chromium (Cr) Micronutrient	Unknown	Blood sugar regulation and can enhance weight gain in livestock.	Deficiency causes diabetes like illness.
Copper (Cu) Micronutrient	Very important in plants reproductive growth stage and indirect role in chlorophyll production. Deficiency results in major yield and quality losses.	Red blood cells and skin pigments, In nature, migrating animals may move from low copper to high copper areas.	Emphysema, high cholesterol, heart and muscle damage when deficient.
Iron (Fe) Micronutrient	Critical for chlorophyll formation and photosynthesis. Important in enzyme systems and respiration in plants.	The oxygen transporter in red blood cells and the red color in muscles.	Lack of iron causes anemia and failure to produce red blood cells. Iron is also necessary for white blood cells in disease immunity responses.
Magnesium (Mg) Macronutrient	The key element in the chlorophyll molecule. There would be no greening in the absence of Mg. First shows up as yellowing on older leaves.	Present in the body skeleton and a co-factor in many enzyme reactions.	Involved in protein synthesis DNA and RNA. Present in all green plant parts that are consumed as food.
Manganese (Mn)	Important for all cereals on high pH mineral (alkaline) and organic soils. Enzyme systems involved with carbohydrate and nitrogen metabolism.	Bones, connective tissue and genetic proteins.	As for animals, including fat metabolism
Molybdenum (Mo) Micronutrient	Essential for nitrogen fixation in legumes and nitrogen metabolism Mo deficiency resembles iron chlorosis. Forages range from 0.1 to 3 ppm/kg of dry matter.	Involved in iron metabolism and enzyme reactions.	Available in grain seeds and animal livers. Seems to be involved in gout and sexual impotence.

Table 4.13-b: Essential minerals with brief descriptions of their roles.

Nickel (Ni) Micronutrient	Role in plants unknown. Present in nuts, beans and peas.	Co-factor for certain enzyme systems.	In the 1970's, evidence that high iron intake increased the need for nickel.
Potassium (K) Macronutrient	The major ion inside every living plant and animal cell.	Involved in nerve impulses and muscle contraction, including the hear t muscle.	With extreme sweating or diarrhea, potassium deficiency can occur (over- use of diuretic medications).
Sodium (Na) Micronutrient	Many cultivated crops, such as beets, were originally sea shore plants. Sugar beets will respond to sodium fertilization.	Major ion in the fluids of the body outside the cells. Present usually as sodium chloride (NaCl).	Sodium controls body water balance and has a role in muscle contraction.
Zinc (Zn) Micronutrient	Very important in corn and bean production. Deficiencies usually occur on eroded soils low in organic matter with high pH Essential for sugar regulation and enzymes that control plant growth.	Important for growth of hair, healing wounds and cell division	Important in taste and as an enzyme detoxifier component for alcohol.

The level of toxic elements found to be very low in all samples of this plant as compared to the essential elements, however some of minerals are needed in relatively very small amounts, while some of them such as Cd, Co and Cr could be toxic for human health and their availability in herbal medicines are controlled as recommended by the WHO guidelines for herbal assessing quality (WHO 2007).

The level of toxic elements found to be very low in all samples of this plant as compared to the essential elements such as Na, K, Mg, Mn, Zn, Co, Cr, and Fe. These essential elements may be directly or indirectly helpful for management of many diseases.

Aluminum and zinc were detected in *C. incana* leaves and they have important role in curing the skin problems. These important elements and their salts are used in skin infections as disinfectant and cleansing agent. The Zn is cofactor for many enzyme required for the healing damaged skin. The level of aluminum was found to be high in all *C. incana* leaves. When paste of leaves is applied on infected skin this may play roles such as antiseptic, soothing and cooling effects (Sahito, Memon *et al.* 2003).

In addition, clinical studies conducted on maternal exposure had shown that aluminum might cause embryo toxicity and affect the developing nervous system in fetus (Paternain, Domingo *et al.* 1988).

The environmental protection agency (EPA) has set the maximum acceptable level of aluminum in fresh water at 750 µg/liter (Kg). In *C. incana* leaves, the result of aluminum was between 557 to 1111 mg/Kg, which means that one cup prepared by 2 grams of the leaves will contain about 1668 µg of aluminum which exceed the maximum acceptable limit (1243.6 µg /2 cups).

Minerals concentration in medicinal plants depends upon geographical location, soil, air and water contamination. In general, the uptake of metals by plants is influenced by a number of factors including metal concentrations in soils, cation-exchange capacity, soil pH, organic matter content, types and varieties of plants and plant age. However, the most important factor is the concentration of the metal in the soil and the existing environmental conditions (Wang, Takematsu *et al*. 2000, Rengel 2004, Annan.k, Dickson. Rita A *et al*. 2013, Losfeld, L'Huillier *et al*. 2014, Karak, Sonar *et al*. 2015).

Finally, the believing that medicinal plants are safe and devoid of toxicity could be misapprehended and therefore, WHO recommended that medicinal plants should be checked for the presence of certain metals which might deteriorate the general health (WHO 2007).

Chapter Five

Conclusions & Future Work

5. Conclusions and Future Work

5.1 Conclusions

This research is the first study to scan the volatile chemical constituents of *C. incana* harvested from different locations in Palestine.

The oil obtained by SD was analyzed by GC-MS and the yield for each sample was calculated based on dry basis. However, The GC-MS technique was utilized and found to be precise, accurate and reliable in the separation and identification of the components of *C. incana* complex volatile mixtures. Seventeen volatile components were separated and identified. The predominant components were pulegone followed by p-menthan-3-one.

The antioxidant activity results revealed that the activity of the essential oil is less than that of the positive control (BHT) and it increased with time. The antioxidant activity of the essential oil was much less than the BHT activity, which may be due to the lower phenolic contents in the plant.

The essential oil antimicrobial experiments revealed activity against both bacteria and fungus. Moreover, it is clear that 5 µl of the tested essential oil exhibits remarkable antimicrobial activity against some gram positive bacteria (*Staphylococcus aureus*) and against gram negative bacteria (*E.coli and Salmonella*).

The observed activity is almost the same as gentamicin in case of *Staphylococcus aureus* as and almost two times more effective than gentamicin in case of *E.coli and Salmonella*. Furthermore, this volume of *C. incana* oil was three times more effective than nystatin in case of *Candida Albicans and Saccharomyces*. This promising result is important due to the increasing resistance against the existing antimicrobial agents in addition to its previously identified toxicity.

Screening of *C. incana* leaves minerals revealed that it is rich in calcium, potassium and magnesium. Therefore, special recommendations for consumers might be necessary in order to prevent *C. incana* minerals from binding with drugs and affecting their absorption.

5.2 Future Work

The following are some suggestions that may be taken into consideration for further future investigation:

1. The present investigation considered *C. incana* essential oils merely at their vegetative stage. The components at different stages of plant life cycle can be examined in order to study the seasonal differences. The same procedure and practice should be applied and compared to the gathered results.
2. The antioxidant activity, bioactivity and toxicity of the essential oils at different stages of the plant life cycle from different extracts can also be systematically explored.
3. Additional work is recommended to estimate the minimum inhibitory concentration (MIC) and the safety of the essential oils.
4. *C. incana* oil effect on broader existing antimicrobial agents is recommended.
5. The effect of synergistic combination of pulegone and p-menthan-3-one in comparison to the oil mixture is recommended.
6. Minerals present in *C. incana* from other different sites is recommended.

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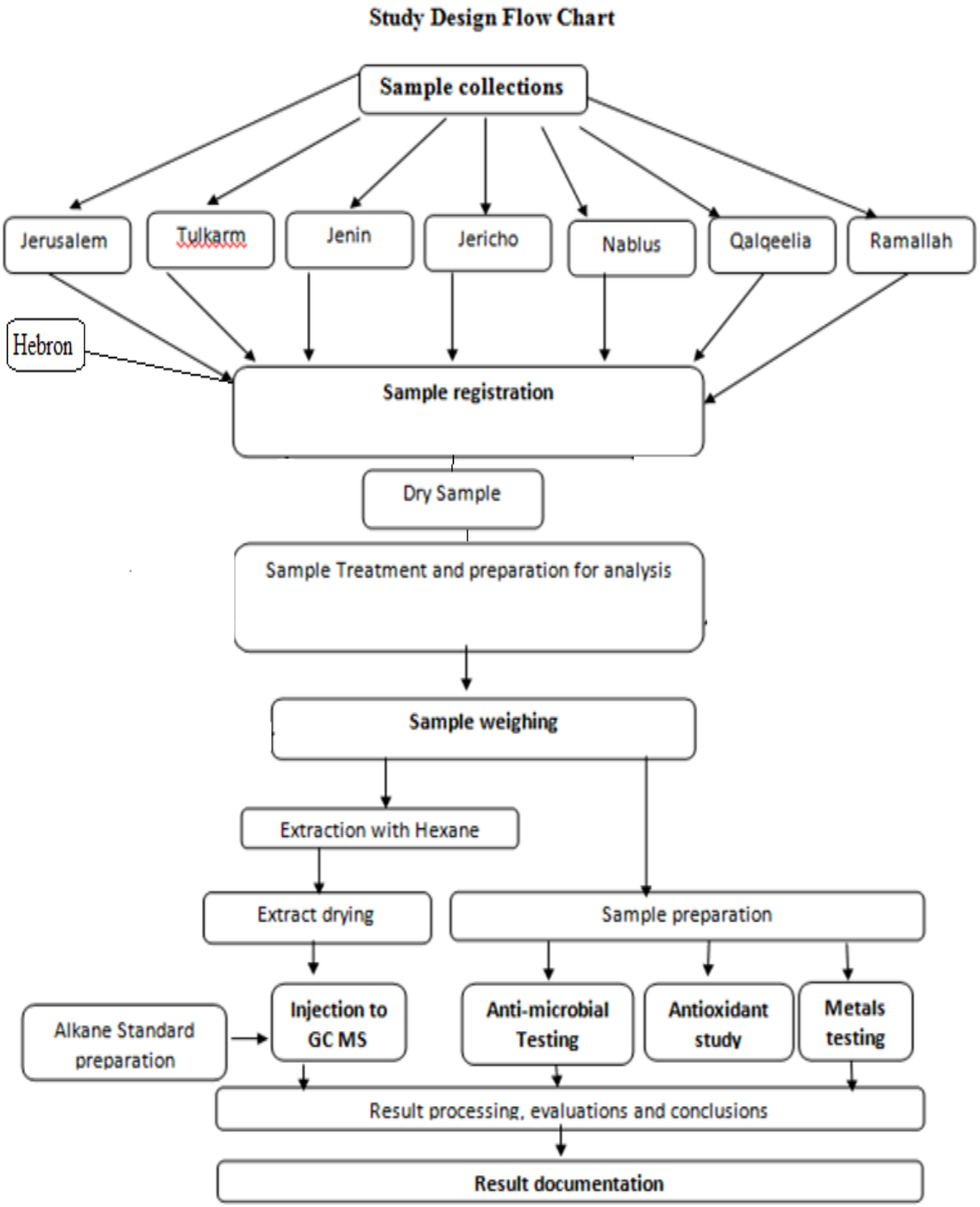
Appendices

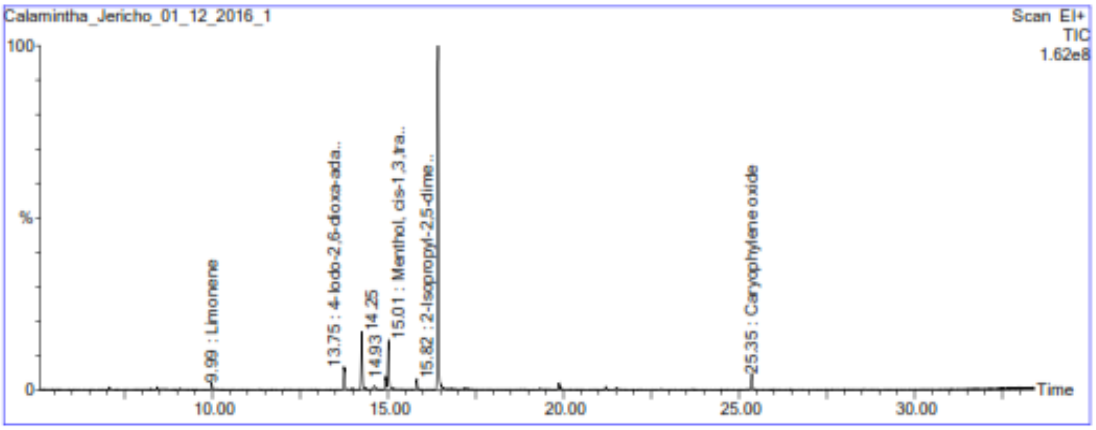
Appendix-1: Study design flow chart



Appendix-2: Samples of TIC for GC-MS Analysis Reports

Qualitative Report

File: C:\TurboMass\FINAL TRANING 2904.PRO \Data\Calamintha_Jericho_01_12_2016_1.raw
Acquired: 01-Dec-16 04:10:14 PM Printed: 24-May-17 03:59 PM
Description:
GC/MS Method: GC: KI Alakane C10-C40 17_4_2013.mth MS: KI Alkane C10-C
Sample ID: Vial Number: 2



#	RT	Scan	Height	Area	Area %	Norm	Name	
1	7.057	556	906,394	26,522.5	0.226	0.39	1S-alfa-Pinene	
2	8.266	669	257,999	7,060.7	0.061	0.11		
3	8.440	936	1,071,736	33,255.7	0.286	0.49	4(10)-Thujene	
4	8.565	973	295,106	12,342.4	0.106	0.18		
5	9.071	1115	305,416	10,926.6	0.094	0.16		
6	9.956	1372	3,243,576	113,513.0	0.976	1.69	Limonene	
7	10.114	1406	166,656	6,574.6	0.057	0.10		
8	11.316	1746	145,667	6,665.3	0.057	0.10		
9	13.756	2431	10,641,269	401,300.9	3.451	5.97	4-Iodo-2,6-dioxa-	
10	13.993	2497	550,671	19,124.1	0.164	0.28	Isomenthone	
11	14.260	2572	27,302,490	1,068,620.6	9.190	####	1R,4R)-(+)-p-Menthan-3-	
12	14.353	2596	791,505	30,190.1	0.260	0.45		
13	14.599	2667	1,446,031	109,396.3	0.941	1.63	Isopulegone	
14	15.012	2783	21,963,545	791,661.7	6.807	####	Menthol, cis-1,3,trans-1,4-	
15	15.624	3011	4,172,546	153,700.2	1.322	2.29	2-Isopropyl-2,5-	
16	16.426	3160	162,069,326	6,723,441.0	57.813	####	Pulegone	

Inst() ACQUISITION PARAMETERS
Oven: Initial temp 50 °C for 2 min, ramp 5 °C/min to 180 °C, hold 0 min, ramp 15 °C/min to 280 °C, hold 5 min, InjAauto=235 °C, Volume=0 µL, Split=20:1, Carrier Gas=He, Solvent Delay=5.00 min, Transfer Temp=240 °C, Source Temp=220 °C, Scan: 50 to 480Da, Column 28.0m x 250 µm

Qualitative Report

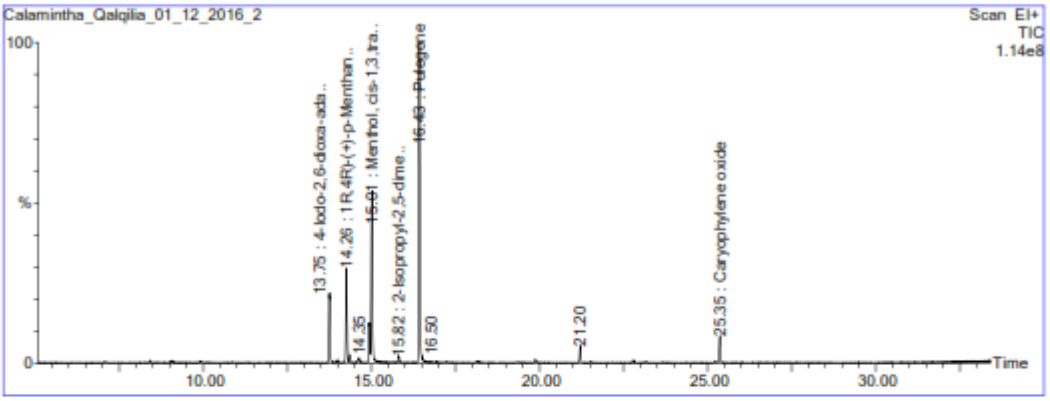
File: C:\TurboMass\FINAL TRANING 2904.PRO \Data\Calamintha_Jericho_01_12_2016_1.raw
Acquired: 01-Dec-16 04:10:14 PM Printed: 24-May-17 03:59 PM
Description:
GC/MS Method: GC: KI Alakane C10-C40 17_4_2013.mth MS: KI Alkane C10-C
Sample ID: Vial Number: 2

#	RT	Scan	Height	Area	Area %	Norm	Name	
17	16.793	3263	410,524	12,942.9	0.111	0.19		
18	17.156	3355	279,667	12,296.2	0.106	0.18		
19	17.263	3415	360,142	13,114.7	0.113	0.20		
20	19.319	3892	337,337	9,356.2	0.080	0.14	Piperitenone	
21	19.667	4146	2,667,143	136,647.1	1.177	2.04	m-Xylene-2,5-diol	
22	21.192	4516	1,363,920	53,099.2	0.457	0.79	B-Caryophyllene	
23	21.506	4606	601,963	51,975.9	0.447	0.77		
24	22.761	4964	291,963	11,229.9	0.097	0.17	B-Cubebene	
25	25.349	5665	7,425,166	306,706.5	2.654	4.59	Caryophylene oxide	
26	30.469	7126	191,324	9,335.1	0.080	0.14		
27	32.916	7610	275,321	5,395.3	0.046	0.08		
28	33.207	7691	305,066	6,512.7	0.056	0.10		
29	33.331	7926	216,599	6,111.6	0.053	0.09		

Inst() ACQUISITION PARAMETERS
Oven: Initial temp 50 °C for 2 min, ramp 5 °C/min to 180 °C, hold 0 min, ramp 15 °C/min to 280 °C, hold 5 min, InjAauto=235 °C, Volume=0 µL, Split=20:1, Carrier Gas=He, Solvent Delay=5.00 min, Transfer Temp=240 °C, Source Temp=220 °C, Scan: 50 to 480Da, Column 28.0m x 250 µm

Qualitative Report

File: C:\TurboMass\FINAL TRANING 2904.PRO \Data\Calamintha_Qalqilia_01_12_2016_2.raw
Acquired: 01-Dec-16 06:23:13 PM Printed: 24-May-17 04:19 PM
Description:
GC/MS Method: GC: KI Alakane C10-C40 17_4_2013.mth MS: KI Alkane C10-C
Sample ID: Vial Number: 3



#	RT	Scan	Height	Area	Area %	Norm	Name	
1	7.054	557	469,606	15,966.3	0.133	0.35	1S-alfa-Pinene	
2	8.441	936	665,052	15,877.3	0.132	0.35	4(10)-Thujene	
3	9.049	1109	547,556	13,479.0	0.112	0.29		
4	9.906	1350	456,629	15,893.0	0.132	0.35	Limonene	
5	10.125	1411	234,169	6,943.6	0.058	0.15		
6	10.563	1624	375,999	15,470.1	0.128	0.34		
7	11.311	1744	202,879	6,601.5	0.055	0.14		
8	13.756	2431	24,952,422	953,507.4	7.914	####	4-Iodo-2,6-dioxa-	
9	13.993	2497	883,363	29,244.9	0.243	0.64	Isomenthone	
10	14.260	2572	33,552,392	1,221,763.0	10.140	####	1R,4R)-(+)-p-Menthan-3-	
11	14.349	2597	2,095,663	70,407.6	0.584	1.54		
12	14.641	2679	1,748,097	104,582.1	0.868	2.28	Isopulegone	
13	15.015	2784	55,676,776	2,277,539.8	18.902	####	Menthol, cis-1,3,trans-1,4-	
14	15.516	3009	2,336,306	84,290.5	0.700	1.84	2-Isopropyl-2,5-	
15	16.425	3160	113,776,424	4,562,004.0	38.026	####	Pulegone	
16	16.521	3207	1,763,376	75,699.7	0.630	1.66		

Inst() ACQUISITION PARAMETERS
Oven: Initial temp 50 °C for 2 min, ramp 5 °C/min to 180 °C, hold 0 min, ramp 15 °C/min to 280 °C, hold 5 min, InjAauto=235 °C, Volume=0 µL, Split=20:1, Carrier Gas=He, Solvent Delay=5.00 min, Transfer Temp=240 °C, Source Temp=220 °C, Scan: 50 to 480Da, Column 28.0m x 250 µm

Qualitative Report

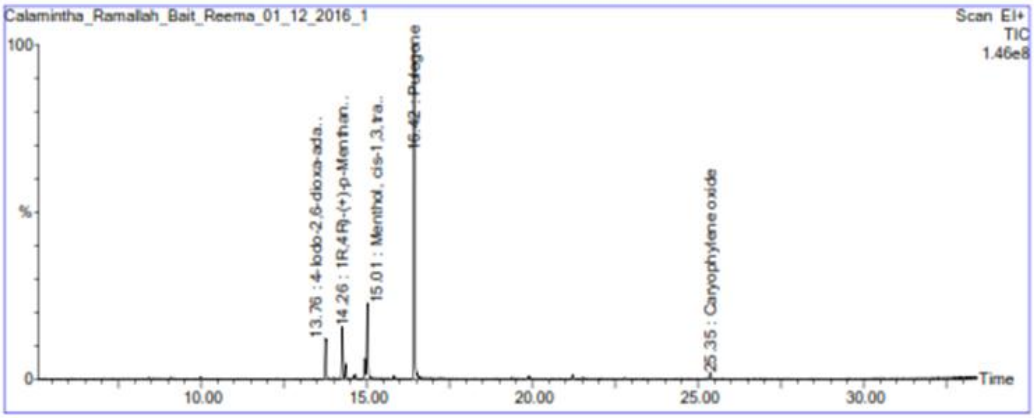
File: C:\TurboMass\FINAL TRANING 2904.PRO \Data\Calamintha_Qalqilia_01_12_2016_2.raw
Acquired: 01-Dec-16 06:23:13 PM Printed: 24-May-17 04:19 PM
Description:
GC/MS Method: GC: KI Alakane C10-C40 17_4_2013.mth MS: KI Alkane C10-C
Sample ID: Vial Number: 3

#	RT	Scan	Height	Area	Area %	Norm	Name	
17	16.942	3325	241,644	10,622.7	0.066	0.23		
18	17.173	3390	191,794	6,206.9	0.052	0.14		
19	18.138	3661	417,614	24,416.6	0.203	0.53		
20	19.331	3996	244,194	21,445.1	0.178	0.47	Piperitenone	
21	19.669	4147	1,126,933	55,347.0	0.459	1.21	m-Xylene-2,5-diol	
22	21.205	4522	5,606,218	217,402.5	1.804	4.74	B-Caryophyllene	
23	21.501	4605	376,956	19,104.6	0.159	0.42		
24	22.145	4786	196,641	7,049.0	0.059	0.15		
25	22.794	4968	911,305	34,504.5	0.286	0.75	B-Cubebene	
26	23.157	5070	394,997	15,299.1	0.127	0.33		
27	23.634	5204	165,127	7,466.3	0.062	0.16		
28	25.219	5649	670,636	24,453.7	0.203	0.53		
29	25.354	5687	9,296,636	384,490.2	3.191	8.39	Caryophylene oxide	

Inst() ACQUISITION PARAMETERS
Oven: Initial temp 50 °C for 2 min, ramp 5 °C/min to 180 °C, hold 0 min, ramp 15 °C/min to 280 °C, hold 5 min, InjAauto=235 °C, Volume=0 µL, Split=20:1, Carrier Gas=He, Solvent Delay=5.00 min, Transfer Temp=240 °C, Source Temp=220 °C, Scan: 50 to 480Da, Column 28.0m x 250 µm

Qualitative Report

File: C:\TurboMass\FINAL TRANING 2904.PRO \Data\Calamintha_Ramallah_Bait_Reema_01_12_
Acquired: 2016_1.raw Printed: 24-May-17 04:21 PM
Description: 01-Dec-16 08:36:11 PM
GC/MS Method:
Sample ID: GC: KI Alakane C10-C40 17_4_2013.mth MS: KI Alkane C10-C[illegible]EXP



#	RT	Scan	Height	Area	Area %	Norm	Name	
1	7.092	559	232,499	6,557.6	0.061	0.11	1S-alfa-Pinene	
2	8.442	936	433,539	13,161.0	0.122	0.23	4(10)-Thujene	
3	9.067	1119	299,382	9,711.9	0.090	0.17		
4	9.992	1373	766,152	26,953.6	0.249	0.47	Limonene	
5	10.117	1406	161,399	5,751.1	0.053	0.10		
6	11.303	1741	127,935	5,023.1	0.046	0.09		
7	13.764	2432	17,392,610	666,726.4	6.169	####	4-Iodo-2,6-dioxa-	
8	13.956	2495	523,420	22,155.3	0.205	0.38	Isomenthone	
9	14.263	2572	22,664,566	901,377.6	8.342	####	1R,4R)-(+)-p-Menthan-3-	
10	14.369	2602	5,676,021	219,466.2	2.031	3.80		
11	14.647	2680	1,937,252	104,435.9	0.967	1.81		
12	15.021	2785	31,294,620	1,219,000.4	11.282	####	Menthol, cis-1,3,trans-1,4-	
13	15.627	3011	1,256,360	41,794.0	0.387	0.72	2-Isopropyl-2,5-	
14	16.425	3179	145,460,512	5,760,750.5	53.499	####	Pulegone	
15	16.646	3241	302,991	13,566.0	0.126	0.23		
16	16.799	3284	276,996	6,638.5	0.061	0.11		

Inst() ACQUISITION PARAMETERS
Oven: Initial temp 50 °C for 2 min, ramp 5 °C/min to 180 °C, hold 0 min, ramp 15 °C/min to 280 °C, hold 5 min, InjAauto=235 °C, Volume=0 µL, Split=20:1, Carrier Gas=He, Solvent Delay=5.00 min, Transfer Temp=240 °C, Source Temp=220 °C, Scan: 50 to 480Da, Column 28.0m x 250 µm

Qualitative Report

File: C:\TurboMass\FINAL TRANING 2904.PRO \Data\Calamintha_Ramallah_Bait_Reema_01_12_2016_1.raw
Acquired: Printed: 24-May-17 04:21 PM
Description: 01-Dec-16 08:36:11 PM
GC/MS Method:

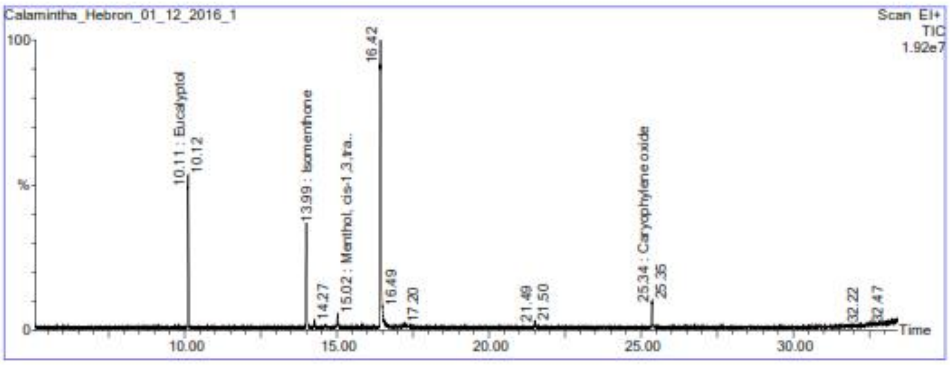
Sample ID: GC: KI Alakane C10-C40 17_4_2013.mth MS: KI Alkane C10-C40 17_4_2013.EXP

#	RT	Scan	Height	Area	Area %	Norm	Name	
17	16.945	3326	205,954	7,052.2	0.066	0.12		
18	17.226	3404	217,057	9,550.6	0.066	0.17		
19	19.366	4005	166,427	11,310.3	0.105	0.20	Piperitenone	
20	19.677	4146	1,163,651	53,935.5	0.499	0.93	m-Xylene-2,5-diol	
21	19.996	4182	140,645	4,756.7	0.044	0.08		
22	21.210	4522	1,865,854	70,522.6	0.653	1.22	B-Caryophyllene	
23	21.496	4603	435,103	18,674.6	0.173	0.32		
24	22.757	4965	377,941	16,244.2	0.150	0.28	B-Cubebene	
25	25.216	5647	205,366	10,547.9	0.098	0.18		
26	25.351	5685	2,637,674	117,991.9	1.092	2.04	Caryophylene oxide	
27	26.566	6586	110,167	3,626.3	0.035	0.07		
28	32.619	7725	165,746	4,261.6	0.040	0.07		
29	33.217	7893	219,956	4,606.2	0.043	0.08		

Inst() ACQUISITION PARAMETERS
Oven: Initial temp 50 °C for 2 min, ramp 5 °C/min to 180 °C, hold 0 min, ramp 15 °C/min to 280 °C, hold 5 min, InjAauto=235 °C, Volume=0 µL, Split=20:1, Carrier Gas=He, Solvent Delay=5.00 min, Transfer Temp=240 °C, Source Temp=220 °C, Scan: 50 to 480Da, Column 28.0m x 250 µm

Qualitative Report

File: C:\TurboMass\FINAL TRANING 2904.PRO \Data\Calamintha_Hebron_01_12_2016_1.raw
Acquired: 01-Dec-16 10:04:50 PM Printed: 24-May-17 05:00 PM
Description:
GC/MS Method: GC: KI Alakane C10-C40 17_4_2013.mth MS: KI Alkane C10-C
Sample ID: Vial Number: 6



#	RT	Scan	Height	Area	Area %	Norm	Name	
1	6.393	363	99,162	3,460.1	0.119	0.38		
2	10.116	1409	10,133,062	340,016.1	11.650	####	Eucalyptol	
3	10.756	1566	116,009	3,587.2	0.123	0.39		
4	12.334	2031	96,551	3,277.2	0.112	0.36		
5	13.556	2374	131,232	3,150.9	0.108	0.34		
6	13.994	2497	6,976,059	273,307.4	9.364	####	Isomenthone	
7	14.266	2574	450,955	20,137.0	0.690	2.20	1R,4R)-(+)-p-Menthan-3-	
8	14.614	2671	167,663	7,443.8	0.255	0.81	Isopulegone	
9	15.020	2785	666,290	34,546.3	1.184	3.77	Menthol, cis-1,3,trans-1,4-	
10	15.625	3011	190,339	6,016.0	0.206	0.66		
11	16.213	3120	176,690	5,541.7	0.190	0.60		
12	16.430	3161	19,074,212	917,219.8	31.426	####	Pulegone	
13	16.594	3227	268,716	6,952.2	0.239	0.76		
14	17.203	3396	235,306	8,650.3	0.296	0.94		
15	21.514	4606	427,546	19,606.2	0.672	2.14		
16	23.206	5063	103,161	2,692.0	0.092	0.29		

Inst() ACQUISITION PARAMETERS
Oven: Initial temp 50 °C for 2 min, ramp 5 °C/min to 180 °C, hold 0 min, ramp 15 °C/min to 280 °C, hold 5 min, InjAauto=235 °C, Volume=0 µL, Split=20:1, Carrier Gas=He, Solvent Delay=5.00 min, Transfer Temp=240 °C, Source Temp=220 °C, Scan: 50 to 480Da, Column 28.0m x 250 µm

Qualitative Report

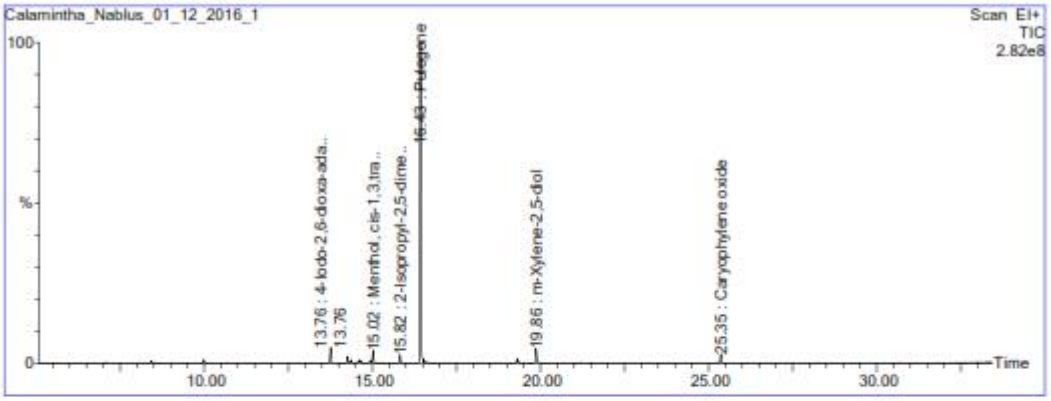
File: C:\TurboMass\FINAL TRANING 2904.PRO \Data\Calamintha_Hebron_01_12_2016_1.raw
Acquired: 01-Dec-16 10:04:50 PM Printed: 24-May-17 05:00 PM
Description:
GC/MS Method: GC: KI Alakane C10-C40 17_4_2013.mth MS: KI Alkane C10-C
Sample ID: Vial Number: 6

#	RT	Scan	Height	Area	Area %	Norm	Name	
17	25.229	5651	91,127	2,711.1	0.093	0.30		
18	25.350	5685	1,644,516	77,474.9	2.654	8.45	Caryophylene oxide	
19	27.741	6356	75,732	2,619.2	0.090	0.29		
20	28.567	6586	116,646	3,603.1	0.130	0.41		
21	29.501	6850	134,577	3,962.9	0.136	0.43		
22	31.560	7426	132,062	4,527.2	0.155	0.49		
23	31.616	7500	125,042	3,177.2	0.109	0.35		
24	32.065	7570	119,472	3,902.6	0.134	0.43		
25	32.792	7774	127,120	2,791.4	0.096	0.30		
26	32.926	7812	164,161	3,367.9	0.116	0.37		
27	33.109	7863	264,992	3,105.3	0.106	0.34		
28	33.220	7894	164,294	4,551.5	0.156	0.50		
29	33.344	7929	179,944	4,664.6	0.160	0.51		

Inst() ACQUISITION PARAMETERS
Oven: Initial temp 50 °C for 2 min, ramp 5 °C/min to 180 °C, hold 0 min, ramp 15 °C/min to 280 °C, hold 5 min, InjAauto=235 °C, Volume=0 µL, Split=20:1, Carrier Gas=He, Solvent Delay=5.00 min, Transfer Temp=240 °C, Source Temp=220 °C, Scan: 50 to 480Da, Column 28.0m x 250 µm

Qualitative Report

File: C:\TurboMass\FINAL TRANING 2904.PRO \Data\Calamintha_Nablus_01_12_2016_1.raw
Acquired: 01-Dec-16 11:33:28 PM Printed: 24-May-17 04:23 PM
Description:
GC/MS Method: GC: KI Alakane C10-C40 17_4_2013.mth MS: KI Alkane C10-C
Sample ID: Vial Number: 7



#	RT	Scan	Height	Area	Area %	Norm	Name	
1	7.094	560	905,220	27,570.6	0.160	0.25	1S-alfa-Pinene	
2	6.269	690	277,403	6,023.4	0.052	0.07		
3	6.440	936	1,495,051	47,256.6	0.309	0.42	4(10)-Thujene	
4	6.565	973	301,299	6,927.9	0.045	0.06		
5	6.721	1017	305,906	10,443.7	0.068	0.09		
6	9.070	1115	341,251	24,440.7	0.160	0.22		
7	9.993	1374	2,620,345	95,665.9	0.627	0.86	Limonene	
8	10.125	1411	367,653	12,254.0	0.080	0.11		
9	13.765	2433	12,266,796	467,739.4	3.169	4.36	4-Iodo-2,6-dioxa-	
10	14.267	2574	5,162,636	179,261.1	1.172	1.61	1R,4R)-(+)-p-Menthan-3-	
11	14.370	2603	2,455,160	92,950.4	0.606	0.83		
12	14.601	2666	2,274,566	65,437.0	0.559	0.77	Isopulegone	
13	14.697	2695	254,365	6,242.6	0.041	0.06		
14	15.021	2786	10,394,766	370,632.6	2.423	3.33	Menthol, cis-1,3,trans-1,4-	
15	15.164	2826	251,147	7,066.3	0.046	0.06		
16	15.623	3011	6,692,713	230,136.4	1.505	2.07	2-Isopropyl-2,5-	

Inst() ACQUISITION PARAMETERS
Oven: Initial temp 50 °C for 2 min, ramp 5 °C/min to 180 °C, hold 0 min, ramp 15 °C/min to 280 °C, hold 5 min, InjAauto=235 °C, Volume=0 µL, Split=20:1, Carrier Gas=He, Solvent Delay=5.00 min, Transfer Temp=240 °C, Source Temp=220 °C, Scan: 50 to 480Da, Column 28.0m x 250 µm

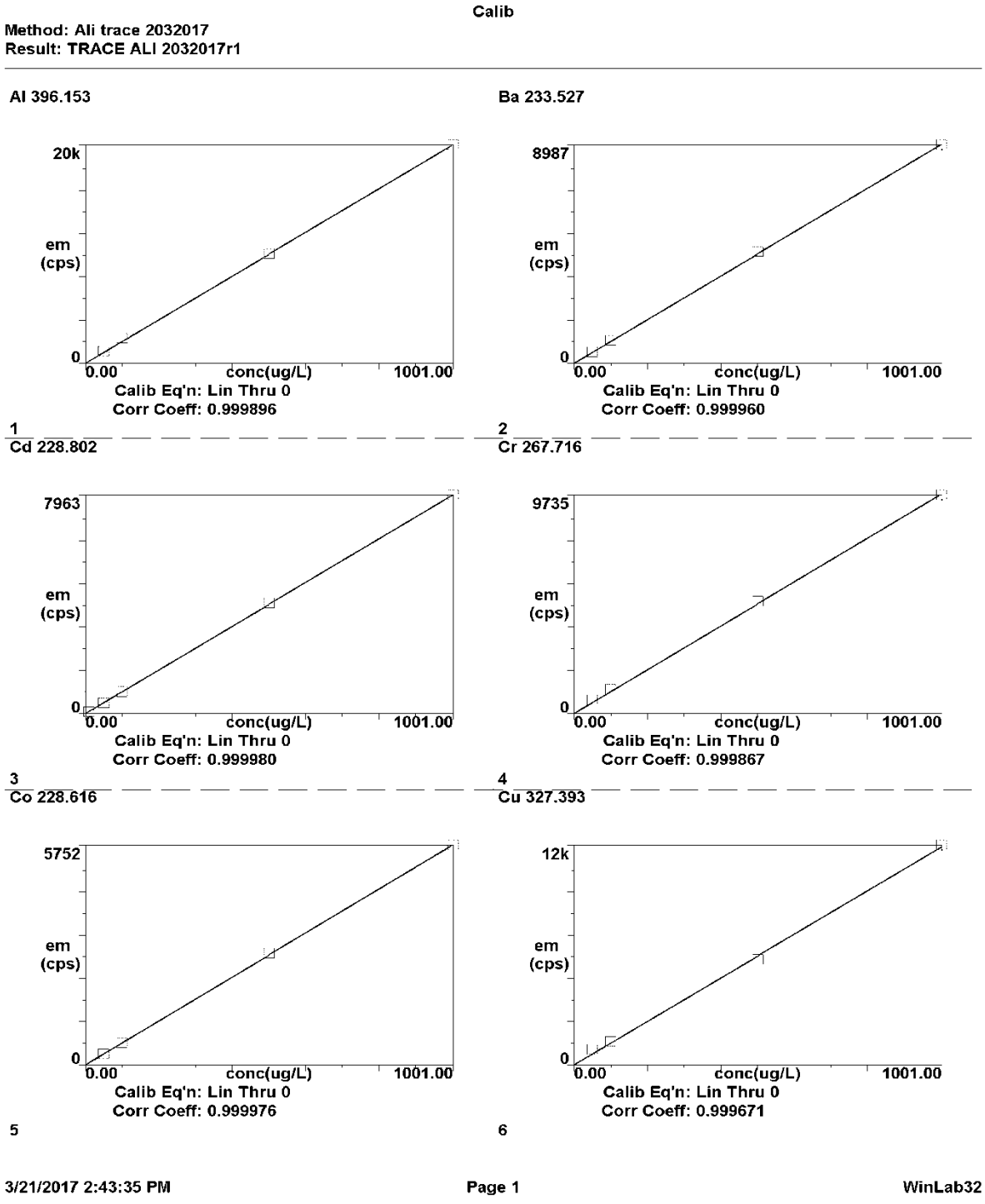
Qualitative Report

File: C:\TurboMass\FINAL TRANING 2904.PRO \Data\Calamintha_Nablus_01_12_2016_1.raw
Acquired: 01-Dec-16 11:33:28 PM Printed: 24-May-17 04:23 PM
Description:
GC/MS Method: GC: KI Alakane C10-C40 17_4_2013.mth MS: KI Alkane C10-C
Sample ID: Vial Number: 7

#	RT	Scan	Height	Area	Area %	Norm	Name	
17	16.426	3161	261,667,744	11,144,317.0	72.670	####	Pulegone	
18	16.649	3243	244,627	10,503.6	0.069	0.09		
19	16.688	3310	217,947	5,716.2	0.037	0.05		
20	17.206	3400	210,447	5,996.3	0.039	0.05		
21	18.621	3853	192,090	6,967.4	0.046	0.06		
22	19.299	3957	3,390,545	146,941.3	0.961	1.32	Piperitenone	
23	19.587	4068	297,557	13,016.5	0.085	0.12		
24	19.662	4145	12,602,133	570,417.5	3.730	5.12	m-Xylene-2,5-diol	
25	21.204	4522	635,359	23,356.5	0.153	0.21	B-Caryophyllene	
26	21.514	4609	426,650	23,554.6	0.154	0.21		
27	23.662	5212	176,659	5,615.9	0.036	0.05		
28	25.236	5654	347,500	14,092.5	0.092	0.13		
29	25.354	5687	7,551,262	299,611.5	1.960	2.69	Caryophylene oxide	

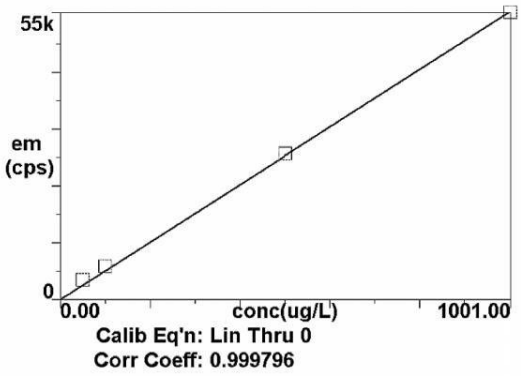
Inst() ACQUISITION PARAMETERS
Oven: Initial temp 50 °C for 2 min, ramp 5 °C/min to 180 °C, hold 0 min, ramp 15 °C/min to 280 °C, hold 5 min, InjAauto=235 °C, Volume=0 µL, Split=20:1, Carrier Gas=He, Solvent Delay=5.00 min, Transfer Temp=240 °C, Source Temp=220 °C, Scan: 50 to 480Da, Column 28.0m x 250 µm

Appendix-3: Standard Calibration Curves for Minerals Analysis main and trace elements.

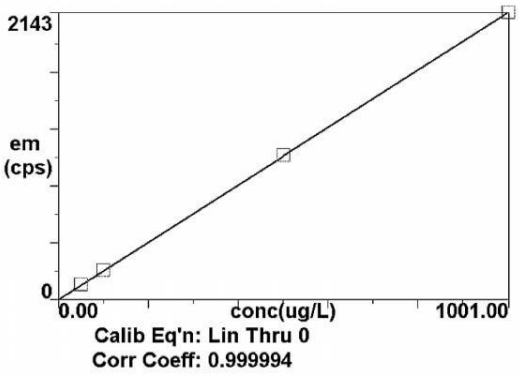


ethod: Ali trace 2032017
esult:

In 257.610

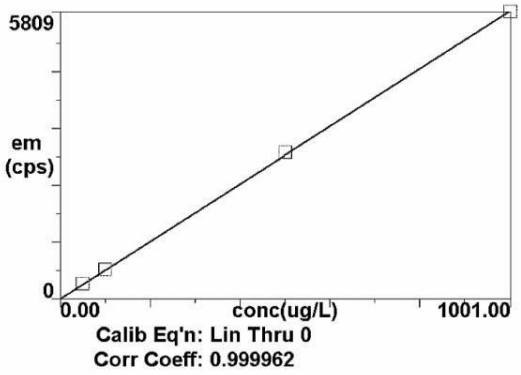


Mo 202.031

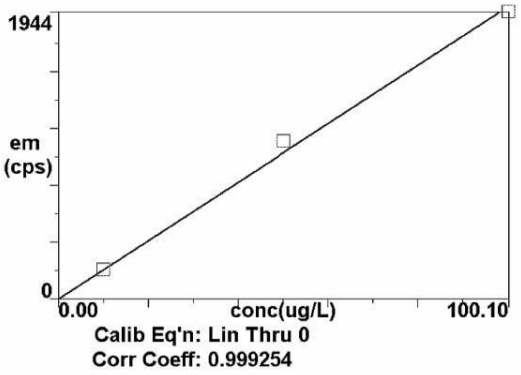


8

li 231.604

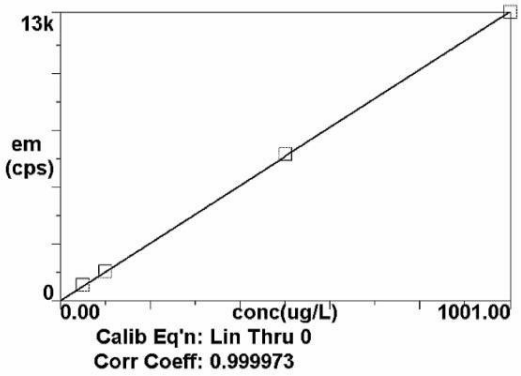


Ag 328.068

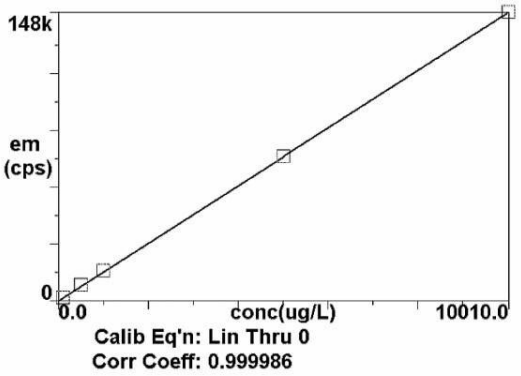


10

n 206.200



Fe 238.204

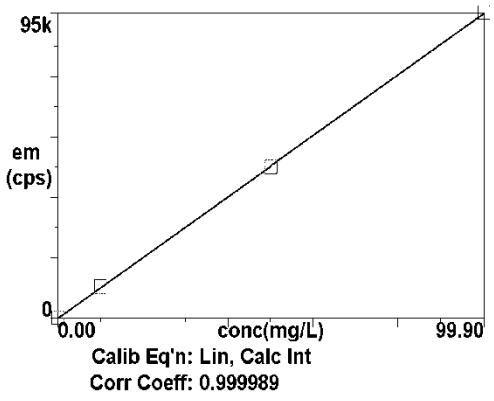


1

12

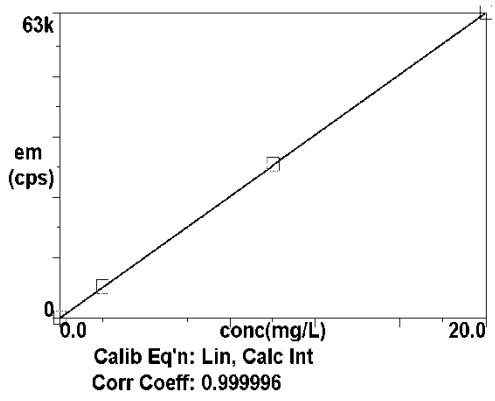
Method: Main Element Ali
Result:

Ca 317.933



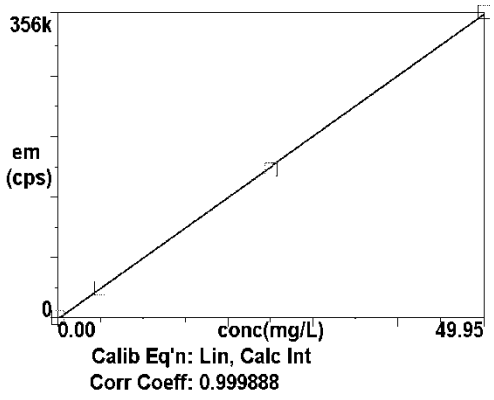
1

Mg 285.213



2

Na 589.592



3

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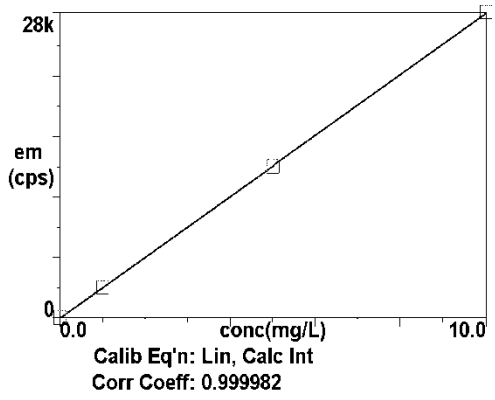

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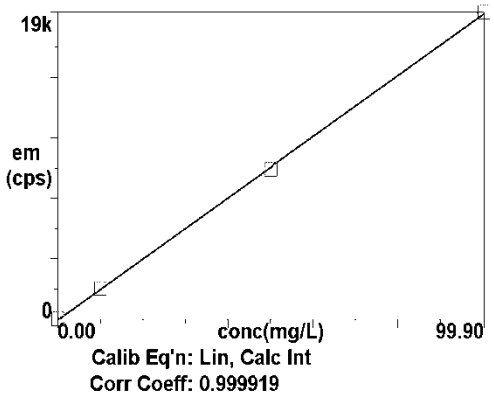

5

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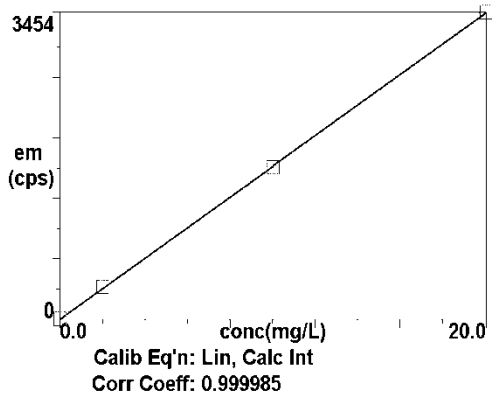

6

تحليل المركبات الثانوية لأوراق الزعتمانة البرية باستخدام جهاز الـ (GC-MS) ودراسة تأثيراتها المضادة للأكسدة والميكروبات

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إشراف: الاستاذ الدكتور صالح أبو لافي

الملخص

إن استخدام الأعشاب الطبية في فلسطين شائع بشكل كبيرو متداول بين الناس. ومن بين الأعشاب التي تستخدم عشبة الزعتمانة. أن استخدام الأعشاب في فلسطين مرتبط بتقاليد متوارثة عن الأجداد أكثر من اعتماده على أسس وأبحاث علمية. وانه من المعروف حاليا ان عشبة الزعتمانة تحتوي على مركبات ثانوية لها استخدامات واسعة في الطب الشعبي وفي صناعة الأغذية كمادة منكهة ومادة حافظة وايضا لها استخدامات في مواد التجميل. الا إن إنتاج ووجود هذه المركبات الثانوية في الزعتمانة يتأثر بالعديد من العوامل المختلفة التي قد تحدد نسبة المواد الثانوية أهمها العوامل البيئية والجغرافية. في الآونة الأخيرة اكتسبت هذه الزيوت اهمية خاصة لكونها مصدرا محتملا للمواد الفعالة حيويا ولكونها أكثر أمانا، خاصة بسبب ظهور مقاومة ميكروبية عند الجراثيم ضد المضادات الحيوية المتوفرة.

في هذا البحث العلمي، تم جمع أوراق الزعتمانة البرية من ثمان مناطق مختلفة في فلسطين، وتم استخراج الزيوت العطرية من الأوراق المجففة بواسطة التقطير المجزأ (Steam Distillation) وقد تم تحديد هوية مكونات الزيت العطري ولأول مرة في فلسطين بواسطة جهاز كروماتوغرافيا الغاز - مطياف الكتلة (GC-MS). وتمت دراسة القدرة المضادة للأكسدة لزيت الزعتمانة باستخدام طريقة (DPPH). وتم دراسة فعالية الزيت العطري ضد ميكروبات مختلفة باستخدام الية القرص. ولتحديد محتوى المعادن في أوراق الزعتمانة الجافة تم استخدام جهاز مطياف الانبعاث الضوئي باستخدام الحث المزدوج البلازمي (ICP-OES).

لقد تم تحديد هوية سبعة عشر مركب أساسي في زيت الزعتمانة، ولقد كانت المكونات الرئيسية في جميع العينات هي البوليجون ثم مينثول باستثناء عينة الخليل والتي كان المكون الرئيسي الثاني فيها هو الاكاليبتول (11.8٪) والذي لم تتجاوز نسبته (0.1٪) في العينات الأخرى كما أن عينة الخليل احتوت

على الايزومينثون بنسبة (9.13٪) والتي كانت نسبة عالية مقارنة مع باقي العينات التي لم تتجاوز في اغلبها (1٪) ويمكن ايعاز ذلك لاختلاف سنة جمع العينة من منطقة الخليل.

لقد لوحظ ان التركيز اللازم من الزيت للوصول الى تثبيط الأكسدة بنسبة 50% (IC_{50}) هو 7.66 ملغم/ مل بعد مرور 30 دقيقة، في حين انه بعد 90 دقيقة أنخفض ليصل الى 4.767 ملغم/ مل، وهذا يشير الى أن النشاط المضاد للأكسدة لزيت الزعتمانة يزداد مع مرور الوقت و مع زيادة التركيز المستخدم من الزيت.

عند دراسة التأثير المضاد للميكروبات ل 5 ميكرو لتر من زيت الزعتمانة تبين أن التأثير المضاد لهذا الحجم من الزيت يفوق تأثير الجنتاميسين في حالة العنقوديات المكورة الذهبية والقولونية والسالمونيلا الا انه اقل من تأثير السيبروفلوكساسين. علاوة على ذلك فأن تأثيره الزيت يفوق مرتين تأثير النيستاتين ضد المبيضات البيض وفطريات الخميرة.

إن أوراق الزعتمانة غنية بالمعادن وخاصة الكالسيوم والبوتاسيوم والمغنيسيوم، وتحتوي على تركيز قليل من الصوديوم مما يجعلها ذات فائدة لمرضى ارتفاع ضغط الدم، ولكن العينة التي تم فحصها لوحظ انها تحتوي على كمية ملفتة للانتباه من الألومنيوم والذي يعرف بأنه يمكن أن يتراكم في الجسم وبالتالي فأنه قد يضر بالصحة. لذا فإنه يوصى بمتابعة العمل على المعادن وذلك بدراسة الأعشاب الطبية والتربة في كل منطقة.